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Reevaluation of granule cells in the cerebellar molecular layer

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Reevaluation of granule cells in the cerebellar molecular layer

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Dedication

To my father, Madhu Sudan Dey, who encouraged me to ignore social expectations and live life as I want to.

To my mother, Malina Rani Dey, who spent the best years of her life raising me. She taught (or, has tried really hard to teach) me love and compassion.

And to my two dogs, Alex and Jason. They have taught me patience and responsibility. I love them both more than anything else.

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My mentor, Hiroshi, asked me what I wanted to work on instead of handing me a project of his liking. From the beginning till the end, he has supported me at every step along the way, sometimes extending to personal difficult times. He made me re-think everything about the way I approach science. He helped me be introspective and improve upon every building block of a good scientist: hypothesis building, experimental design, data collection, analysis, and strong work ethic. Perhaps the more important lessons from Hiroshi were on how to be a better human. I am a work in progress.

Jeffrey Gross was the Associate director at UT Austin when I started graduate school. He gave me extra semester to find my best fit and I landed in the Nishiyama lab, which is where I met my wonderful lab mates Matasha, Alex, Kirthan, and Naoko. Without Matasha's help, qualifying exams would have been a nightmare. And without Naoko's help, I would not be able to test some of the most exciting possibilities of my project. Kirthan helped lighten the workload and at times worked through his weekends as a college student. Besides making lab a really fun place to be, Alex and Matasha made me feel like their family and made me laugh more than I had ever before. They made graduate school one of the best times in my life.

Daniel Johnston listened to my talk and pointed me to electrophysiology and then gave me a whole rig room to do experiments in. I had not patched a single cell before. The entire Johnston/Brager lab helped me throughout the process. Darrin and Rick patiently guided and helped me with experimental design and experiments. Rick has solved every technical difficulty I faced, and there were many. On days when I stayed a

little too long, Rick left me tacos for dinner before he left for the evening. I am not sure how I got so lucky.

Before I came to UT Austin, I have been fortunate to have met and worked for two wonderful scientists, Dr. Amelia Eisch and Dr. Christine Garcia. They have both been huge reasons I have continued in science even with the many hiccups along the way. I worked in the Eisch lab as a Summer Undergraduate Research program participant. Amelia's enthusiasm for neuroscience is contagious and her support during difficult times has been critical in my progress. I worked in Christine's lab as a research associate before I got accepted to the ICMB program. She taught me how to work hard to get something in science. My first publication was from her lab.

I would like to sincerely thank my committee members Dr. Seema Agarwala, Dr. Michael Drew, Dr. Michael Mauk, and Dr. Hitoshi Morikawa for their continuous support and guidance. I am thankful for their insight (collective and individual) and eventual push to create a story out of my findings. My time in graduate school has been as much about getting to know very kind and generous scientists as the cerebellar circuitry.

My friends: Laura, Zeinab, and Rashin who have been there for me during some of the toughest times in the last few years. My sister, Jaba, constantly reminded me that I am not just a reflection of failed experiments. I am not sure if I would be able to make it through without their support.

Abstract

Reevaluation of granule cells in the cerebellar molecular layer

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Neuronal migration to the exact destination is one of the critical steps in the development of the brain. Incomplete or incorrect migration yields ectopic neurons, which can cause neurological defects at varying degrees. However, granule cells in the molecular layer (mGCs) of the cerebellar cortex may challenge this traditional view of ectopic neurons. Around birth, granule cell precursors proliferate near the pia mater and then migrate down to the granule cell layer, though the molecular layer. But some granule cell-like cells stay in the molecular layer even in normal adult animals. These mGCs were named ectopic granule cells nearly 50 years ago but remain mostly uncharacterized. Here, we have examined them in the molecular layer with a specific marker for mature granule cells and transgenic mice in which they are labeled with a fluorescent protein. Contrary to the previous assumption that mGCs are negligible, we have found that mGCs are among the most abundant neurons in the molecular layer, constituting approximately 30% of the entire cell population. They are produced during a similar period as regular granule cells (rGCs), and *in vivo* time-lapse imaging has revealed that mGCs are stably present in the molecular layer. Whole-cell patch-clamp recordings have shown that mGCs discharge action potentials similar to rGCs, but mGCs and rGCs likely receive different types of excitatory inputs. These results suggest that mGCs are not negligible ectopic neurons, and they might have a unique functional role in the cerebellum.

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Chapter 1: Introduction

The cerebellum plays a crucial role in fine motor control, balance, and motor learning (De Zeeuw 2020). It is suggested to use an error-based supervised learning rule, in which a motor error signal drives plastic changes in cerebellar synaptic circuits to refine motor outputs (Ohyama et al. 2003; Raymond, Lisberger, and Mauk 1996). In addition, studies using human patients with cerebellar lesion strongly suggest cerebellar contributions to emotion and intellectual abilities, expanding the role of the cerebellum to non-motor function (Schmahmann 2019). Due to extensive study of the cerebellum for over a century, it is one of the most morphologically and physiologically well-characterized parts of the brain. Nevertheless, our understanding of cerebellar circuitry keeps evolving with the discovery of new cell types, connectivity, and plasticity (Balmer and Trussell 2019; Guo et al. 2016; Lainé and Axelrad 1994; Mugnaini, Sekerková, and Martina 2011; Schilling et al. 2008; Witter et al. 2016).

The cortical structure, cellular layout, and synapses of the cerebellum was first described in detail by Chan-Palay and Palay in 1974. It was decades before revisions were made in the neuronal and synaptic makeup of the cerebellar cortex and two examples are the discovery of the unipolar brush cell and candelabrum cells (Balmer and Trussell 2019; Mugnaini et al. 2011). Another example of a neuronal population in the cerebellar cortex which have been left uncharacterized are the granule cells in the molecular layer (mGCs) of the adult cerebellar cortex. So far, mGCs have been speculated to be ectopic granule cells which failed to complete migration because they started their migration late and got

stuck in the molecular layer *en route*, thus implying that mGCs are ectopic neurons formed as a result of defective migration (Palay, Chan-Palay 1974; Ponti, Peretto, and Bonfanti 2006). Another prevailing assumption about mGCs is that they do not form any synaptic connection with other neurons, and therefore, do not have a functional role in the cerebellum. Although, there is no evidence to support these speculations so far, mGCs have been dismissed as negligible, erroneous neurons in the cerebellar cortex.

From my observations and others, mGCs have their dendrites in the molecular layer (Lafarga and Berciano 1985; Palay, Chan-Palay 1974). The molecular layer of the cerebellum is densely packed with axons of regular granule cells (parallel fibers), climbing fibers, less densely with the axons of molecular layer interneurons (basket and stellate cells), and occasionally Purkinje cell (PC) axon collaterals (Apps and Hawkes 2009; D'Angelo 2018b; Guo et al. 2016; Hashimoto and Hibi 2012; Witter et al. 2016). Therefore, it is possible that mGCs receive input from one or more of the axons present in the molecular layer.

My dissertation is on the analysis of mGCs. In the following chapters, I will first show the anatomical and developmental characteristics of mGCs. Briefly, I have found that mGCs make up about one third of the molecular layer cell population, one of the most abundant cells in the molecular layer. They are not negligible, erroneous neurons as assumed previously. The peak of mGC proliferation and differentiation is slightly delayed compared to regular granule cells (rGCs). However, the difference is only a few days, and both mGCs and rGCs are born within the first three postnatal weeks. I will next show the

physiological properties of mGCs, the first-ever electrophysiological recordings made from them. I have found that they discharge action potentials in a similar fashion to rGCs, suggesting that they can transmit synaptic signals. Lastly, I will show that mGCs receive excitatory synaptic input and that a higher proportion of their synaptic input shows paired-pulse depression. Only climbing fibers, which do not synapse onto rGCs, show paired-pulse depression in the molecular layer; thus, mGCs might form a previously unknown excitatory circuit in the cerebellar cortex.

In the next few pages, I will discuss general neuronal migration, effects of defective neuronal migration, and then highlight some neuronal types in the brain which are not very well characterized yet, and mostly still ignored. Some of the neurons are dismissed as ectopic neurons even though they were discovered and described during the early days of morphological characterization of the brain. Other neurons, such as the candelabrum cells, are very recent discoveries (Schilling et al. 2008).

Next, I will discuss the tight physical and molecular synchronization of events that take place to form the cerebellar cortex, and then discuss the cortical and synaptic structure of the adult cerebellum. Lastly, I will explain the modular organization of the cerebellum and how each module might be specific to a particular aspect of sensorimotor learning. It is important to characterize each cell type of the cerebellum because our understating of its synaptic and functional layout is constantly evolving (De Zeeuw 2020). I will use rGCs and mGCs as abbreviations throughout to differentiate between the two populations and

use ‘granule cells’ when it is not possible to differentiate between the two, specially while discussing the development of the cerebellar cortex.

1.1 Neuronal Migration

General neuronal development and migration

Neuronal migration, along with neural stem cell proliferation and differentiation into mature neurons or glia, and proper synaptogenesis are critical in the development of the central nervous system. Neurons begin this process in the embryonic stages and continue till the early postnatal days. Most neurons of the neocortex are derived from the walls of the ventricular and subventricular zones which migrate to their destinations to form the cortical layers (Buchsbaum and Cappello 2019; Kriegstein and Noctor 2004). Neuronal migration and cortical patterning seem to occur in waves of migration throughout the embryonic and postnatal days in mice. The cortical layers form in a reverse order where neurons that form successively later during the embryonic days, will move past cells that established layers earlier, and form layers in the more superficial part of the cortex (Angevine and Sidman 1961). This inside-out pattern of neural generation is true for both of the major neuronal populations, excitatory and inhibitory, in the cerebral cortex (Miller 1985). But, excitatory and inhibitory neurons seem to have different stem cell origins and migration trajectories (Kriegstein and Noctor 2004). The initiation of cortical pattern formation is observable as early as embryonic day 11 (E11) in the mouse (Angevine and

Sidman 1961). Broadly, the neurons first migrate tangentially to their approximate prospective location in the embryonic cortex and then form six distinct cortical layers via a combination of chain migration (such as in the olfactory bulb formation) (Lois and Alvarez-Buylla 1994) and radial migration (Buchsbaum and Cappello 2019). The entire process is affected by a multitude of factors, cell intrinsic and external, which work together to form the adult brain.

Neuronal migration defects

And any deviation in the tightly regulated migration patterns will result in severe consequences like prenatal or postnatal death. Survivors will suffer from physiological and functional consequences resulting in a broad category of malformations of cortical development, with neuronal migration disorders being a subcategory of its own (Buchsbaum and Cappello 2019). Neuronal migration defects can arise from genetic, cell and molecular, and or physiological defects (Buchsbaum and Cappello 2019). It is thought that most of the neuronal migration disorders have a genetic origin, but several are also caused by prenatal insults (Roberts 2018). Diseases which originate from migration defects are classified in five general malformations: focal cortical dysplasia, heterotopia, lissencephaly, schizencephaly, and polymicrogyria (Roberts 2018). The resulting clinical manifestations come in various forms but some of the classifications are dysmorphic features, developmental delay, intellectual disability, and epileptic seizures (Buchsbaum and Cappello 2019). The malformation subtypes and their manifestations have their own

sub-classification and range of severity. In some cases, the patients affected by any of the neuronal migration disorder malformation subtypes can die during childhood, as in the case of *Walker-Warburg Syndrome* which is a result of Lissencephaly (Roberts 2018). Because of the variety and severity of problems caused by neuronal migration defects, any ectopic neurons or apparently ectopic neurons, are studied mostly in a disease context (Guerrini and Parrini 2010; Roberts 2018; Wu et al. 2014).

Ectopic neurons in the normal brain

The normal adult brain has at least three regions where ectopic neurons exist and they are the hilus of the dentate gyrus, layer *IV* of the entorhinal cortex, and the cerebellar molecular layer (Canto, Wouterlood, and Witter 2008; Chan-Palay 1972; Gaarskjaer and Laurberg 1983; Pierce, McCloskey, and Scharfman 2011; Scharfman, Goodman, and McCloskey 2007).

Ectopic hilar granule cells: The dentate gyrus in mammals has a discrete layer of granule cells and this layer is very compact (Scharfman et al. 2007). Ramon y Cajal first suggested in 1911 that there were potential granule cells in the hilus of the normal hippocampus, but he used Golgi staining for his anatomical descriptions which made it impossible to assert their identity or numbers. Eventually these cells were named ectopic granule cells and were rarely studied in the context of the normal adult hippocampus. These ectopic hilar granule cells have similar cell body shape, dendritic and axonal projection profiles, membrane properties, synaptic properties compared to regular granule cells

(Gaarskjaer and Laurberg 1983; Scharfman et al. 2007; Scharfman, Goodman, and Sollas 2000). Later studies shifted towards studying them in a disease context, specifically seizures. Several studies have shown that inducing seizure in rodent models increases adult neurogenesis in the hippocampus and one of the effects is an increase in the number of hilar granule cells (Scharfman et al. 2000). The resulting higher number of ectopic hilar granule cells after seizure induction in experimental animals may result in those animals being further predisposed to epileptic seizures (Pierce et al. 2005). Thus, ectopic hilar granule cells have been mostly studied in the context of induced seizures where the number of ectopic granule cells increase artificially afterwards (McCloskey et al. 2006; Pierce et al. 2005, 2011).

Ectopic pyramidal cells in *Lamina dissecans*: The entorhinal cortex is made up of six layers and the two layers which do not have cell bodies (or are very sparse) are layers I and IV(Witter 2007). Layer I is the *molecular layer* and layer IV is the *lamina dissecans*. Layer IV is generally identifiable by the lack of cell bodies and this is the current understanding of the entorhinal cortex architecture. Ramon y Cajal's student, Rafael Lorente de Nó was the first to propose the terms to define the layers of the entorhinal cortex in 1933 and he considered layer IV (under current nomenclature) to be an extension of layer III with sparse neurons (Larriva-Sahd 2014; Witter 2007) Therefore, historically, the *lamina dissecans* was acknowledged to have neurons. However, in the current scientific literature, any mention of these neurons does not exist except in reviews by Menno P. Witter (Canto et al. 2008; Witter 2007). There are sparse pyramidal shaped neurons in the

lamina dissecans and, according to unpublished data from Witter lab, these neurons have morphological and physiological characteristics similar to the pyramidal neurons in III and V (Canto et al. 2008). In addition, they also report the presence of bipolar cells which have a spindle like shape. Neither of these cell types in *lamina dissecans* have been characterized enough to be published.

Cerebellar molecular layer granule cells: Early investigators did not use any staining method that was specific to granule cells in their morphological study of the cerebellum (Lafarga and Berciano 1985). But it was noted that granule cells are easily identifiable, even under a light microscope, because of their dense heterochromatin in the nucleus. Due to the non-specific nature of Golgi staining which they used for overall identification of cell types; they could identify very few granule cells in the molecular layer. Their observations were mostly limited to the more easily identifiable conical shaped clusters of granule cells with the base flat against the pial surface. They dismissed granule cells in the molecular layer (mGCs) as ones that could not complete their migration to the internal granule cells layer. As a result, mGCs' physiology and synaptic connections were not studied. Later, these mGCs were only studied in the context of a disease model (Yamanaka and Obata 2004). But in 2008, Ponti and Bonfanti showed that rabbits have a second layer of cells right under the pial surface of the molecular layer and it emerges around puberty and after the proliferative external granule cell layer is exhausted at around five weeks (Ponti et al. 2006). This subpial layer continues to proliferate and eventually differentiate to new neurons until about 6 months (Ponti et al. 2006). Most of these new

neurons are interneurons but a few granule cells were close to the subpial layer and some in the molecular layer around months 3 and 4 (Ponti, Peretto, and Bonfanti 2008). The authors had done immunohistochemistry on cerebellar sections at different ages of the rabbits to hypothesize that the granule cells near the sub pial layer and in the molecular layer were newly born cells from the proliferative subpial layer and were on their way to the granule cell layer.

Molecular layer granule cells seem to be present in the adult cerebellum across several species, at least in mammals from published and unpublished data, including from our lab. A 1952 paper shows non-specific staining of an adult human cerebellar section and the molecular layer shows several small cells with intensely stained chromatin (a criterion used by Chan-Palay to identify cerebellar granule cells) along with clusters close to the pia with a similar morphology and dark nucleus (Brzustowicz 1952). In addition, data from our lab show that there are considerable numbers of mature granule cells in the molecular layer of both mouse and rat.

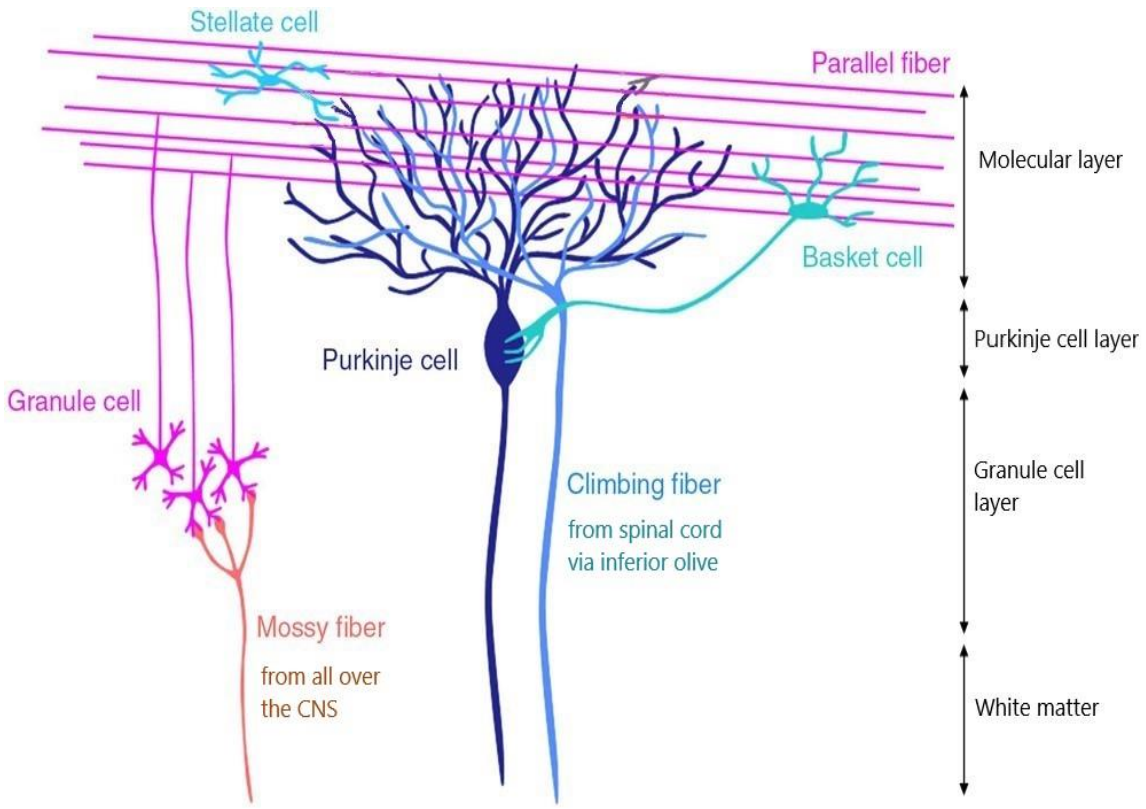

Figure 1.

Simplified version of the cerebellar cortex.

The topmost layer is the molecular layer, followed by the Purkinje cell layer, granule cell layer, and the white matter. The molecular layer is densely packed with parallel fibers which are granule cell axons. The molecular layer also has climbing fibers, axons of inferior olivary neurons, and Purkinje cell dendrites. The two known cells of the molecular layer are the basket and stellate cells. The granule cells receive input from mossy fibers. Adapted from Hirano and Kawaguchi, 2014.

Granule cell development and migration

Timing and source: The adult cerebellar cortex has three distinct layers: the most superficial is the molecular layer, right underneath is the PC layer, which is a unicellular layer, and beneath it is the granule cell layer. Cerebellar rGC make up more than half of the entire neuronal population of the adult brain and they communicate information from the mossy fibers to the PCs (Wingate and Hatten 1999). I will discuss the cortical organization of the cerebellum and the various inputs and outputs in later sections of the introduction. Figure 1 shows a simplified version of the cerebellar cortex. In my discussion of the timing and events of cerebellar development, I will focus on the major milestones relevant to the development of rGC destined for the granule cell layer.

The rhombic lip of the hindbrain in embryos gives rise to the cerebellum (Wingate and Hatten 1999). In vertebrate embryos, the hindbrain, or rhombencephalon, sits right beneath the midbrain, or mesencephalon. In the mouse, the rhombencephalon is a transient state and is apparent from embryonic day 8.5 (E8.5) to E9.5 (D. Morales and Hatten 2006). The rhombencephalon is made up of the anterior metencephalon and the posterior myelencephalon. The metencephalon is called rhombomere 1 and the myelencephalon is called the remaining rhombomeres. The anterior rhombencephalon in the mouse E9.5 embryo still has a dorsal fissure, called the rhombic groove or rhombic fossa, and it will be closed soon (D. Morales and Hatten 2006). It is also referred to as the rhombic lip because of the shape. At the rhombic lip, the alar plates of the anterior portion run bilaterally to form a wide V-shape and they are interconnected at the dorsal portion by the roof plate

(Wullimann 2011). The upper rhombic lip (URL) is the source of the cerebellum and it's nuclei (Alder, Cho, and Hatten 1996; Hallonet, Teillet, and Le Douarin 1990; Wang, Rose, and Zoghbi 2005; Wullimann 2011). Around E10 in mouse the two germinal zones in the URL become apparent, they are the anteriorly located ventricular proliferative zone and the germinal trizone which is located at the small posterior end of the rhombic lip (Goldowitz 1998; Wullimann 2011). Later on the germinal trizone became known as the URL (Wullimann 2011). The ventricular proliferative zone and URL are both adjacent to the 4th ventricle.

The ventricular proliferative zone generates precursors of cerebellar neurons in waves. The cerebellar nuclear neurons originate from the ventricular proliferative zone around E10, next the PC precursors are formed around E11 to E13 (Goldowitz 1998). By E14, the PC precursors migrate radially to from the ventral zone to their putative location (D. Morales and Hatten 2006). This migration of PCs is guided by the processes of radial glial cells which become Bergmann glia upon maturation (Yuasa et al. 1996). The radial glia cell bodies align themselves on the surface of the 4th ventricle and their processes extend to the pia of the immature cerebellum and PCs originating on specific regions of the ventricular zone migrate radially along these processes while PCs originating on other regions of the ventricular zone take a different route (Rahimi-Balaei et al. 2018; Yuasa et al. 1996). PCs keep proliferating and form clusters as the cerebellum continues to expand during development, and they start forming their typical regularly spaced monolayer after birth, around postnatal day 3 (P3) (Rahimi-Balaei et al. 2018).

The external granule layer (EGL) is the source of all granule cells of the cerebellar cortex (Goldowitz 1998; D. Morales and Hatten 2006; Wang et al. 2005). The EGL starts to form at the URL around E11 and is obvious by E13, and covers the entire pial surface of the emerging cerebellar cortex by E17/18.5 by travelling rostromedially (Alder et al. 1996; Goldowitz 1998; Wang et al. 2005). During this time, the granule cell precursors (GCPs) proliferate rapidly and migrate tangentially to make the EGL (Rahimi-Balaei et al. 2018). The EGL is a highly proliferative zone and the GCPs stay strictly mitotic until around birth (Espinosa and Luo 2008). The GCPs stay mitotically active until postnatal week 3 and the EGL persists until then; some GCPs start to differentiate around birth and migrate to the nascent granule cell layer, and it starts to become obvious around P5 (Espinosa and Luo 2008). This postmitotic migration to the granule cell layer is radial and guided by the Bergmann glial fibers which have their cell body right below/in the now developing PC layer (Rahimi-Balaei et al. 2018). Figure 2 is an illustration of the major events which take place during cerebellar development around birth, starting from E18 when the PC precursors have migrated to the nascent cerebellar cortex and are in the cluster stage.

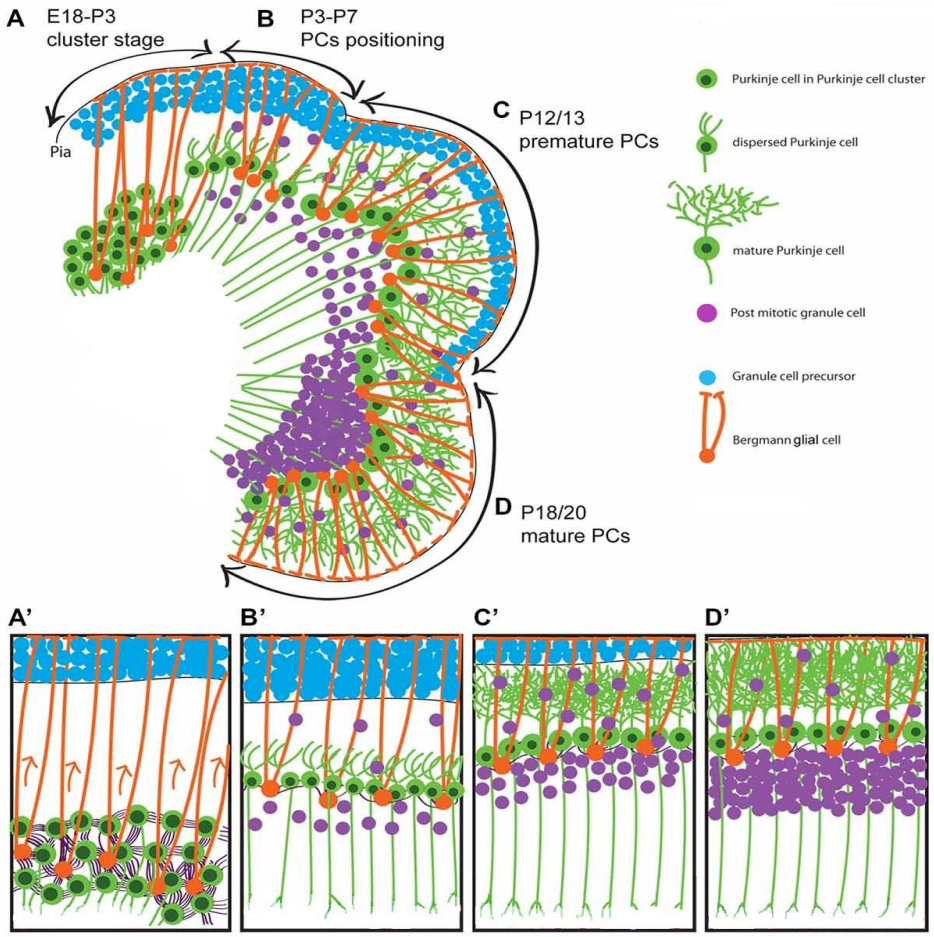

Figure 2.

Neuronal migration during postnatal development.

Illustration of cerebellar development viewed from the sagittal section of embryonic and postnatal mice. Purkinje cells (PCs) cluster disperse to form PC monolayer and start maturation while granular layer forms from the external granule cell layer, a proliferative zone (E18–P20). (A, A′) Around E18 to P3, Bergmann glial cell bodies are located close to PCs during the clustering stage and extend their fibers to the cerebellar pial surface. (B, B′) PCs are in the dispersing and positioning process (P3–P7) to form monolayer and show shortened Bergmann glial fibers. (C, C′) Shows how premature PCs (P12/13) are arborized while granule cell precursors (GCPs) migrate to the developing granular layer and become mature granule cells. (D, D′) Around P18/20 is the end stage of the PCs maturation and GCPs differentiation and migration to the granule cell layer. P, postnatal day; E, embryonic day. Adapted from Rahimi-Balaei *et al*., 2018.

Major Signaling molecules involved: Cerebellar development is simultaneous with the development of the entire nervous system; it is a complex process and involves many signaling pathways working together (Goldowitz 1998). In this section, I will focus on the major signaling molecules and pathways that have been clearly implicated as being essential to the formation of the granule cell layer. A few molecules are necessary for the formation of the cerebellum (Goldowitz 1998; Lewis et al. 2004; Rahimi-Balaei et al. 2018; Wang et al. 2005). The very early on in embryonic development, around E9, the rhombomere 1 roof plate induction is started by the bone morphogenic protein (BMP) signaling pathway and the downstream transcription factor LIM Homeobox Transcription Factor 1 Alpha (*Lmx1a*) is essential for the formation of the anterior roof plate over/of rhombomere 1 (Chizhikov 2006). The anterior roof plate formation is necessary for formation of the cell population on the URL which will eventually generate granule cells (Chizhikov 2006; Wang et al. 2005). By E9.5 in the URL, a subset of cells start to express the basic helix-loop-helix transcription factor Mouse atonal homolog 1 (*Math1*/ *Atoh1*) and all granule cells are derived from this population (Ben-Arie et al. 1997; Wang et al. 2005). *Math1* is expressed in the EGL into the postnatal days and stops during the GCP to granule cell maturation and inward migration to the granule cell layer (Ben-Arie et al. 1997).

The early EGL is multilayered with the top most layers being most mitotically active and the lower layers consisting of mostly cells undergoing differentiation and starting migration (Espinosa and Luo 2008). In the EGL, GCPs proliferate rapidly in the embryo and continue to do so after birth (mostly in the top EGL layers post birth), up to

postnatal day 14/15 but reducing the rate exponentially with time after birth, with no GCP proliferation by P18 (Espinosa and Luo 2008; Fujita 1967). One of the crucial molecules needed for the expansion of GCPs on the EGL, both in the embryonic and postnatal ages, is Sonic hedgehog (*Shh*) secreted by the PCs located right underneath the EGL in the embryo (Lewis et al. 2004; Dahmane and Ruiz-i-Altaba 1999; Wallace 1999). PCs start secreting *Shh* starting at E17.5 and continuously secrets *Shh* until P5, slows down the secretion but continues to do so until P14 (De Luca et al. 2016; Dahmane and Ruiz-i-Altaba 1999). *Shh* is essential in multiple pathways in the developing embryo (and therefore) conditional knockout of *Shh* from the PCs reveal that it is important for GCP proliferation but not for GCP differentiation to mature granule cells or their migration to the granule cell layer (Lewis et al. 2004). It is hypothesized that GCP proliferation is driven by transient (approximately E12 to E17) autocrine signaling (*Shh* from GCP) before PCs take over *Shh* secretion at E17.5 (Dahmane and Ruiz-i-Altaba 1999).

During the postnatal days, the number of PCs regulate the EGL thickness; the proliferative activity of the EGL relies on the PC population but the number PCs is not dependent on GCP or granule cell population (Goldowitz 1998; Lewis et al. 2004; Dahmane and Ruiz-i-Altaba 1999). However, proper PC dendrite formation is dependent on number and maturation of granule cells (Pan et al. 2009). External addition of *Shh* to GCPs will prevent them from differentiating and continue to proliferate (De Luca et al. 2016). These observations suggest that GCP proliferation is dependent upon Shh secretion

by PC and therefore is limited to about P14 in mice, which is when PCs stop secretion *Shh*, as discussed above.

Shh secreted by PCs drives GCP proliferation by binding to its receptors, patched (*Ptc*), expressed by EGL cells, mostly on the topmost proliferating layers (Wallace 1999; Wang and Liu 2019). Patched is a trans-membrane receptor and inhibits smoothened (*Smo*) and *Smo* is a G-protein coupled receptor (Wang and Liu 2019). *Shh* binding on *Ptc* relieves the inhibition on *Smo* and this eventually activates *Gli1* (Dahmane and Ruiz-i-Altaba 1999; Wallace 1999; Wang and Liu 2019). *Gli1* is a part of a group of transcription factors that are required for cell cycle progression, and so the activation of *Gli1* in the outermost EGL layers leads to GCP proliferation.

GCP proliferation continues to slow down after birth and completely absent by P18 (Espinosa and Luo 2008) and part of the reason is decreasing *Shh* secretion by PCs. But, the exact pathway to stop *Shh* secretion from PCs, and GCP differentiation to mature granule cells is still under investigation (Wang and Liu 2019). An interesting finding is that *Shh* induces the maturation of radial glia to Bergmann glia and granule cells migrate radially along the Bergmann glia processes (Espinosa and Luo 2008; Dahmane and Ruiz-i-Altaba 1999). The structural integrity of the Bergmann glia is needed for the proper radial migration of granule cells and these glial cells also secrete factors (*Wnt3*) which stops GCP proliferation (by targeting the *Shh* pathway) (Xu et al. 2013). Therefore, Bergmann glia maturation is a potential part of the granule cell differentiation process.

The other major signaling molecule necessary for GCP proliferation, and induction of EGL progenitors, is *Math1*, and it is expressed until postnatal period on the external layers of the EGL where the GCPs are undergoing rapid transit amplifying proliferation (Ben-Arie et al. 1997). *Neurod1* is a basic helix-loop-helix transcription factor which is expressed in differentiating granule cells in the inner EGL; these cells are differentiating, with occasional expression on outer EGL (Pan et al. 2009; Shiraishi et al. 2019). The maturation of granule cells seem to be, at least, partly driven by the upregulation of *Neurod1* and downregulation of *Math1* in a gradient manner on the EGL, correlated to the proliferation to differentiation gradient; and the *Neurod1* downregulates *Math1* (Pan et al. 2009).

These recent findings on the molecular interactions necessary for GCP to proliferate, dependence on *Shh* from PCs and downregulation of *Math1* by *Neurod1*, further support anatomical finding that GCP proliferation timeline is restricted to about P14/18. By P21, the EGL is non-existent and none of the precursor marking genes are expressed in granule cells (Espinosa and Luo 2008; Shiraishi et al. 2019). However, Chan-Palay had hypothesized that the mGCs are granule cells that were stuck on their migratory path to the granule cell layer and the molecular layer is not their destined location. At the time, knowledge of the role of Bergmann glia in the migration of granule cells was limited to a physical scaffold for the radial migratory pattern. There is recent evidence to suggest a much more involved role of radial and Bergmann glia in cerebellar development (Araujo, Carpi-Santos, and Gomes 2019; Xu et al. 2013). In particular, molecular cross-talk between

Bergmann glia, it's maturation, and granule cell migration to the granule cell layer is becoming more evident (Araujo et al. 2019; Weller et al. 2006).

1.2 Cerebellar Cortex

Neuronal organization

The cerebellum is made up of the cerebellar cortex and the cerebellar nuclei (Hirano and Kawaguchi 2014). The adult cerebellum is longitudinally divided in five zones; the middle portion is the vermis, the paravermis are two smaller regions on either side of the vermis, and the most lateral regions are the two hemispheres (Beckinghausen and Sillitoe 2019). From the surface, the cerebellum appears highly folded and the sagittal section through the vermis reveals ten distinct and major lobules, each separated by a fissure of varying depths (Beckinghausen and Sillitoe 2019). Each major lobule is further folded on the surface and the grooves are called sulci. The topmost cortical layer is the grey matter, which is followed by the internal white matter which is mostly a dense network of fiber tracts, along with three pairs of cerebellar nuclei which are on either side of the midline and together they form the deep cerebellar nuclei (DCN) (Beckinghausen and Sillitoe 2019; Cohen 2013). These three pairs of DCN are the only output of the cerebellum (except from the vestibular cerebellum) and they are called fastigial, interpositus, and dentate nuclei, going from medial to lateral part of the cerebellum (Cohen 2013; D'Angelo 2018b). The

vestibular cerebellum is the exception to the rest of the cerebellum and projects directly to the vestibular nuclei in the brainstem (D'Angelo 2018b; Ito 2006).

The DCN neurons project to 1) various brainstem nuclei, and 2) the cerebral cortex (Baumel 2009; D'Angelo 2018b). The DCN neurons receive input from 1) cerebral cortex, 2) various brainstem nuclei, and 3) spinal cord (Baumel 2009; Beckinghausen and Sillitoe 2019; Cohen 2013; D'Angelo 2018b). Although the DCN has several sources of input, the most significant contribution is from the PC axons of the cerebellar cortex and therefore PCs are the primary output of the cerebellar computations (Baumel 2009; Wagner and Luo 2020).

The cerebellar cortex receives input via two pathways: 1) mossy fibers bring in information from the brain and spinal cord through the nuclei in the brainstem and pons, and 2) climbing fibers bring in information from the spinal cord and they are the axons of the inferior olivary nucleus in the brainstem.

The topmost molecular layer consists the dendrites of PCs that arborize in the sagittal plane, the axons (parallel fibers) of rGCs, climbing fiber axons, Bergmann glial fibers, Golgi cell dendrites, basket cells, and stellate cells (Apps and Hawkes 2009; D'Angelo 2018b; Hashimoto and Hibi 2012). The PC layer is a monolayer of cells with Bergmann glia cell soma at a similar plane; rodents have about 160,000 to 600,000 PCs in their cerebellum (Apps and Hawkes 2009; Korbo et al. 1993; Qu and Smith 2005; Xu et al. 2013). The granule cell layer is very densely packed with rGCs along with Golgi cell bodies interspersed in addition to Lugaro cells and unipolar brush cells (UBCs); UBCs are

mostly found in the vestibulo-cerebellum (van Dorp and De Zeeuw 2014; Hashimoto and Hibi 2012; Warren and Sawtell 2016).

Purkinje cells have one or two primary dendrites which arborize in the molecular layer, the arborization fans out on the sagittal plane but is flattened in the perpendicular direction (Bower 2015; Ruigrok, Sillitoe, and Voogd 2015). These dendritic arbors receive excitatory input from climbing fibers and parallel fibers, which are axons of rGCs, and inhibitory input from stellate and basket cells (Hirano 2018; Hirano and Kawaguchi 2014; O'Donoghue, King, and Bishop 1989; Ruigrok et al. 2015; Witter 2007; Zhou et al. 2020). Among these inputs to the PCs, the parallel fiber inputs are the most numerous, 80,000 to 200,000 inputs to each PC (Bower 2015; Ito 2006). PC axons go through the granule cell layer and have several collaterals and they are also oriented in the same sagittal plane, with some exceptions, as the dendritic tree (Ruigrok et al. 2015; Witter et al. 2016). PC are the sole output of the cerebellar cortex and their major axonal synapse is on the DCN neurons; PC output is inhibitory because PCs are GABAergic neurons (Baumel 2009; Hashimoto and Hibi 2012; Oertel et al. 1981; Witter et al. 2016). These inhibitory PC collaterals also terminate onto Lugaro cells, stellate cells, basket cells, other PCs, rGCs, and potentially the much less characterized candelabrum cells which reside just below the PC layer (Guo et al. 2016; Hirono et al. 2012; Ruigrok et al. 2015; Schilling et al. 2008; Witter et al. 2016).

Climbing fibers are one of the two major inputs to the cerebellar cortex. Each inferior olivary neuron contributes to 6 to 10 climbing fiber axons with an average of 7 and each climbing fiber from a single IO neuron can terminate in multiple lobules but are

restricted to narrow parasagittal zones (Sugihara, Wu, and Shinoda 2001). Each of the climbing fiber will arborize and make extensive glutamatergic synaptic connections with PC dendrites, about 250 to 1,400 synaptic boutons on each; making single climbing fibers to PC synapse one of the most powerful synaptic connections in the brain (Hansel, Linden, and D'Angelo 2001; Ito 1984; Otis 1997; Streng, Popa, and Ebner 2017). Few climbing fiber collaterals provide input to the DCN directly but the behavioral significance is unknown (D'Angelo 2018b; Hansel et al. 2001). Climbing fiber to PC synaptic input is a massive glutamatergic input and shows an all or none complex spike on the PC, and brief but sustained 5 Hz input from climbing fiber to PC can cause long term depression (LTD) on the PCs (Hansel and Linden 2000). A complex spike starts off as a (regular) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and Na^+ mediated action potential which spreads passively, but then the resulting Ca^{++} influx causes little spikelets on top of the action potential (Hansel and Linden 2000; Hansel et al. 2001; Stuart and Häusser 1994). The relevance of LTD in cerebellar motor learning will be discussed in later sections.

Mossy fibers are the second of the two major inputs to the cerebellar cortex which bring in information from all over the CNS and convey this synaptically to rGCs. Parallel fibers are the axons of rGCs in the granule cell layer and each ascends to the molecular layer and bifurcates at a plane right angled to the PC dendritic arborization (Chan-Palay 1972; Ruigrok et al. 2015). The mossy fibers convey this information to a large number of rGCs and this is a major signal diverging point in the CNS (Hoxha et al. 2016). This signal

divergence is an important aspect of pattern separation of incoming sensory information via mossy fibers and this information is conveyed to PCs via parallel fibers (to be discussed further in later section) (Kalmbach, Ohyama, and Mauk 2010). Parallel fibers make glutamatergic synapses on PCs, which is mediated by AMPA receptors, and generates a simple spike on PCs (Hoxha et al. 2016; Ito 1984; Ruigrok et al. 2015). Simple spikes are conventional action potentials and the PC fire them spontaneously or can be induced by parallel fibers (Ruigrok et al. 2015). Parallel fiber to PC input can result in either long term potentiation (LTP) or LTD, and is bi-directional, depending on the circumstances (Coesmans et al. 2004a; Hansel et al. 2001; Hirano 2018; Hoxha et al. 2016; Ruigrok et al. 2015). The large number of parallel fibers (over 100,000) bringing in signal from rGCs to PCs makes the parallel fiber-PC synapses large signal converging points in the CNS (Hansel and Linden 2000; Hoxha et al. 2016; Ito 1984). Parallel fiber input alone to PCs results in LTP induction, for a short period of time (Coesmans et al. 2004a; Sakurai 1987). However, when the PCs receive parallel fiber input in conjunction with climbing fiber input at low frequencies in the range of 4 Hz, i.e equivalent to a scenario where both mossy fiber and climbing fiber inputs are coming into the PCs, LTD occurs at the parallel fiber-PC synapse (Sakurai 1987). The induction of both LTP and LTD at the parallel fiber-PC synapse is dependent on calcium transients in the PC, but at different concentration thresholds (Coesmans et al. 2004b; Sakurai 1990). The all or none climbing fiber-PC complex spike dependent on Ca^{++} transient in the PC is needed for the parallel fiber-PC LTD induction (Sakurai 1990) and hence the requirement for simultaneous climbing fiber

and parallel fiber input to the PCs for LTD induction. Although simultaneous climbing fibers input is needed for parallel fiber-PC LTD induction, a prior climbing fiber-PC LTD will reduce the chances of parallel fiber-PC LTD induction on the same PC (Coesmans et al. 2004b).

The PCs also receive inhibitory input from molecular layer interneurons, basket and stellate cells; both interneuron types are GABAergic, basket cell axons synapse on the PC soma and stellate cells synapse on the PC dendrites (D'Angelo 2018b; Ruigrok et al. 2015). Together, there are about 10 molecular layer interneurons for each PC and they receive both climbing fiber and parallel fiber input, like the PCs nearby (Ekerot and Jörntell 2001; Hirano and Kawaguchi 2014; Korbo et al. 1993; Sugihara, Wu, and Shinoda 1999; Suter and Jaeger 2004). As the excitatory inputs excite the PC, the same inputs will excite the molecular layer interneurons which will, in turn, inhibit PCs ; thus causing feed forward inhibition (Mittmann, Koch, and Häusser 2005; Santamaria, Tripp, and Bower 2007). Feed forward inhibition on PCs is very fast, in the range of 1 to 5 milliseconds and it allows for precise spike timing in PCs by removing transient parallel fiber inputs (Santamaria et al. 2007; Suter and Jaeger 2004). This precise timing of PC spikes due to feed forward inhibition is important because PCs are the sole output of the cerebellar cortex and inhibit DCN neurons. This inhibitory input from molecular layer interneurons to PCs can also undergo plasticity and has implications in cerebellar motor learning (Hirano 2018; Hirano and Kawaguchi 2014; Tanaka et al. 2013).

Deep cerebellar nuclei (DCN) neurons can be classified according to their somatic and dendritic morphology or their use of neurotransmitters (glutamate, GABA, or glycine) (Sultan, Czubayko, and Thier 2003; Uusisaari and Knöpfel 2011). DCN neurons receive input from climbing fiber collaterals, mossy fiber collaterals, and PC axons; but the largest portion of input to the DCN neurons is GABAergic input from PCs, comprising of at least over 50% of all inputs to the DCN (D'Angelo 2018b; Palkovits et al. 1977; Uusisaari and Knöpfel 2011). The DCN neurons form two major pathways of information flow, one within the cerebellum and one that takes information outside the cerebellum (there is some cross-talk between these two pathways) (Bengtsson and Hesslow 2006; Lu, Yang, and Jaeger 2016; Palkovits et al. 1977; Uusisaari and Knöpfel 2011). Within the cerebellum, the pathway is from inferior olive-climbing fiber collaterals to PCs and back to inferior olive via DCN neurons and this is called the olivo-cortico-nucleo-olivary (OCNO) loop; the OCNO loop seems to follow the modular pattern of the cerebellum, by topography and zebrin patterning (Buisseret-Delmas and Angaut 1993; Sugihara and Shinoda 2007; Sugihara et al. 2001). The output of the cerebellar cortex is via the second pathway mentioned above, which flows information from the inferior olive to the DCN neurons via the PCs and it is referred to as the corticonuclear projection; the PCs follow the same compartmentalized projection to DCN neurons in this system as well (Buisseret-Delmas and Angaut 1993).

These DCN relay the cerebellar information to three main regions; the cerebellar cortex, the cerebral cortex, and the spinal cord via the premotor nuclei (Loutit, Vickery,

and Potas 2020; Uusisaari and Knöpfel 2011). The cerebellar information is relayed via several premotor structures such as the dentate nuclei, red nucleus, and thalamic nuclei (Houck and Person 2015; Loutit et al. 2020). The major DCN output pathway is to the cerebral cortex via the thalamic pathway (Loutit et al. 2020; Lu et al. 2007). The projections to the cerebral cortex include the motor cortex in region M1 and area 46 of prefrontal cortex (Kelly and Strick 2003).

Granule cells are between the mossy fiber terminals and PCs (Palay, Chan-Palay 1974). The cerebellar rGCs are the most numerous cells in the brain with a ratio of 270 to 420 rGCs to each PC in the rodent, and totaling to about 92 million rGCs (Harvey and Napper 1988; Korbo et al. 1993). They have a very small soma with an average of three to four dendrites ending in a claw-like shape (Palay, Chan-Palay 1974). Their axons arise from the soma, or often from the proximal end of a dendrite, and ascend to the molecular layer, past the PC layer, where they bifurcate to become parallel fibers (Palay, Chan-Palay 1974). Parallel fibers are unmyelinated and the thinnest axons of the brain, with an average diameter of 0.16 μm and can be as low as 0.1 μm (Perge et al. 2012; Vranesic et al. 1994). Being so thin and unmyelinated, the action potential conduction speed of parallel fibers is unsurprisingly the lowest among axons, at 0.25 m/s (Vranesic et al. 1994). Even with the extremely thin parallel fibers, they still make up about 35% of the molecular layer by volume (Harvey and Napper 1988). The extreme thinness of parallel fibers makes them difficult to trace but an approximate average length of the parallel fiber, in entirety of bifurcation, is 5 mm (Harvey and Napper 1988). Parallel fiber axons run perpendicular to

the PC dendritic arborization and each parallel fiber makes an average of 675 synaptic connections on PC dendrites on either side of its path; but it only makes synaptic connections with about half of the PC dendrites in its path (Harvey and Napper 1988; Napper and Harvey 1988). As mentioned in prior sections, each PC dendrite can have about 150,000 parallel fiber synaptic connections, but, approximately 85% of those connections do not generate electrical responses *in vitro* (Isope and Barbour 2002; Napper and Harvey 1988). The *in vitro* results agree with *in vivo* data where a majority of parallel fiber-PC synapses are also silent (Ekerot and Jörntell 2001). This means that even though the PC dendrite has beams of parallel fibers synapsing on them, only a small fraction of the parallel fiber-rGC input is needed to get a response from PCs. In fact, only about 150 parallel fibers, which have above threshold synaptic strength, are needed to be simultaneously activated to drive a PC (Isope and Barbour 2002). The exact number of parallel fiber needed to excite a PC may be slightly different if input from inhibitory interneurons is also taken into consideration (Isope and Barbour 2002).

Granule cells receive synaptic input from mossy fibers (J Altman 1982; Palay, Chan-Palay 1974). Each mossy fiber terminal makes one synapse with one rGC and each rGC receives three to five mossy fiber synapses (D'Angelo et al. 1995a; Ito 1984; Palay, Chan-Palay 1974). Each rGC will fire an action potential only after at least two to three of the mossy fiber synapses are activated, each mossy fiber elicited excitatory postsynaptic potential sums linearly in the rGC (D'Angelo et al. 1995a; Ekerot and Jörntell 2008). *In vivo* work has shown that mossy fiber input to a granule cell, although from different mossy

fiber, is from the same modality or somatosensory receptive field (Jorntell and Ekerot 2006). In addition, the mossy fibers innervation specific rGC (or small set of rGCs) are not only from the same modality, but also code the sensory information in the same (specific) way (Bengtsson and Jorntell 2009; Jorntell and Ekerot 2006). In the cerebellum, mossy fibers seem to distribute the same signal to as many rGCs as possible and individual rGC receive the same mossy fiber input from different sources (Ekerot and Jörntell 2008). This redundancy of incoming signal to the rGCs is important because it helps to reduce the signal-to-noise ratio of sensory inputs to the cerebellum; mossy fibers are known to fire at about 40Hz at rest and rGCs help to relay only relevant information to the PCs (Jorntell and Ekerot 2006; Perge et al. 2012).

Golgi cells are interspersed in the granule cell layer with their axons in the granule cell layer and dendrites fanning out in the molecular layer; they are GABAergic interneurons (Heine, Highstein, and Blazquez 2010; Palay, Chan-Palay 1974; Simpson et al. 2005). There are over 5,700 rGCs to each Golgi cell in the granule cell layer (Palkovits, Magyar, and Szentágothai 1971). The Golgi cells axons branch extensively and each cell can inhibit a large number of rGCs, in the range of 1000s of rGC; but, the exact number is unknown because the Golgi cell axons branch extensively and is difficult to trace and so have not been reconstructed completely (Barmack and Yakhnitsa 2008; Palkovits et al. 1971; Tabuchi et al. 2019). Golgi cell axons terminate on the glomeruli where mossy fiber afferents terminate in a rosette shape and synapse onto rGCs (Palay, Chan-Palay 1974). There, Golgi cells provide rGCs with fast inhibitory postsynaptic potentials, spill-over

mediated inhibitory component, and tonic GABA mediated inhibition (Tabuchi et al. 2019). Golgi cells receive input from rGCs via the parallel fibers and from mossy fibers and therefore can provide rGCs with feedforward inhibition (Heine et al. 2010). These attributes of Golgi cell connectivity provide rGCs with global inhibition and changes GC firing thresholds by spill-over mediated feedforward inhibition (Tabuchi et al. 2019).

The redundancy of mossy fiber inputs combined with the global and feedforward inhibition by Golgi cells makes input to the rGCs sensitive and sparse making the granule cell layer suitable for pattern separation (Cayco-Gajic, Clopath, and Silver 2017; Tabuchi et al. 2019). Modeling studies show that a sparse input to the granule cell layer can improve learning speed and robustness in the cerebellum (Cayco-Gajic et al. 2017).

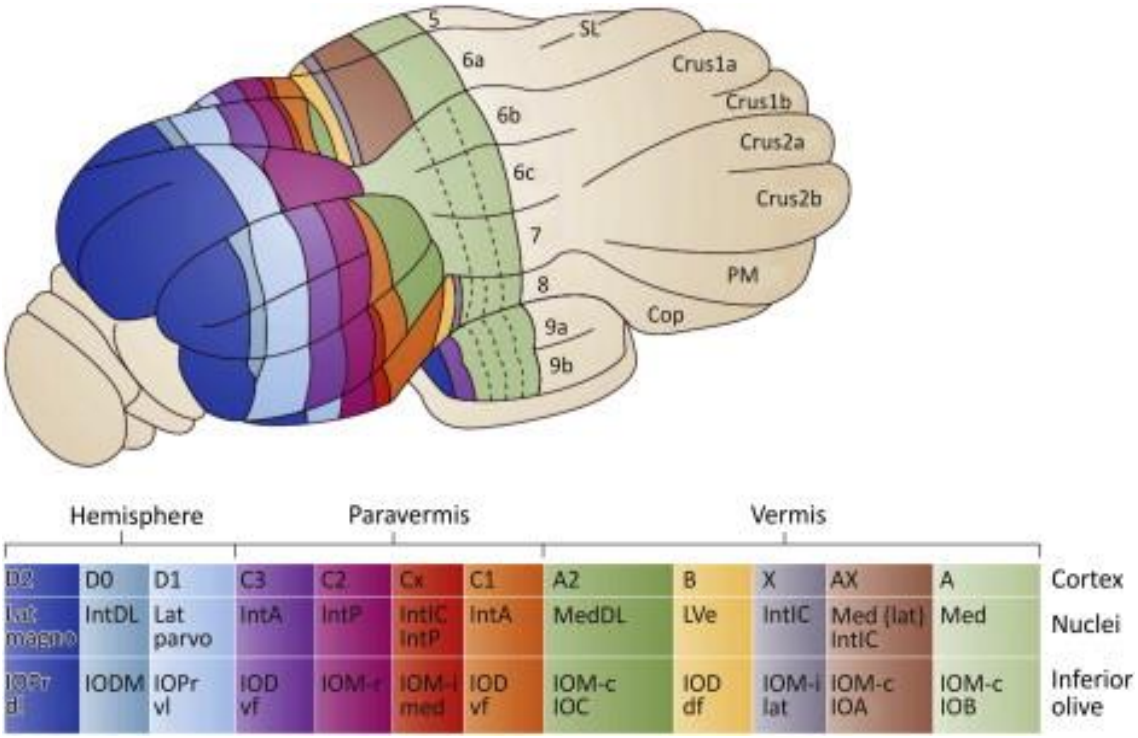

Figure 3.

Modular organization of the cerebellum.

Dorso-caudal perspective of the modular organization of the cerebellar cortex delineated approximately. The longitudinal modules/zones are color coded on the cerebellar surface and schematically represented in the box. The box shows the olivocortical and corticonuclear connections to the corresponding part of the contralateral inferior olivary complex supplying climbing fibers to the zone and the part of the cerebellar nuclei that receives the afferents from these zones. Adapted from book chapter by Voogd *et al*., 2015.

Modular organization of the cerebellum

The cerebellar cortex is organized in rostrocaudally oriented modules and each module can be further divided into microzone; each module is defined by a group (generally, longitudinally arranged) of PCs which receives climbing fiber input from a well-defined group of inferior olivary neurons and the PCs, which in turn, project to a specific set of DCN neurons (Bengtsson and Jorntell 2009; Buisseret-Delmas and Angaut 1993; Cerminara and Apps 2011; Ruigrok et al. 2015; Sugihara and Shinoda 2007; Sugihara et al. 2001). Figure 3 shows the modular organization of the cerebellum. The longitudinal zones are color coded and the box shows the corresponding areas of the IO and DCN neurons. As described above, even the climbing fiber collaterals synapsing on to the deep cerebellar nuclei follow the modular organization, visualized by the ZebrinII expression pattern (Apps et al. 2018; Apps and Hawkes 2009; Fujita and Sugihara 2013; Voogd et al. 2003). Because of the modular nature of the cerebellar cortical circuitry, the PCs within a module show similar complex spike pattern, due to the spontaneous activity coupling of IO neurons (via gap-junctions) and possible similarity in the physiological nature of PCs within a module (Apps and Hawkes 2009; Sugihara, Marshall, and Lang 2007). There is evidence that the climbing fibers (strong glutamatergic axons) release glutamate for varying lengths of time in different modules, hence showing further segregation in the modules (Paukert et al. 2010). Each of the cerebellar modules controls some aspect of fundamental motor or behavioral function; for example, head and eye movement is controlled by module A of the vermis (Cerminara and Apps 2011).

The fundamental modular architecture is also respected by Golgi cell soma and its dendrites in the molecular layer, where they stay within the bounds of a module (Golgi axon tracing was difficult and unreliable in the study, as is generally the case for Golgi axons) (Sillitoe et al. 2008). Basket cells are inhibitory interneurons of the molecular layer and they wrap their descending axonal projections around the PC soma and initial segment of the PC axon hillock, this is called a pinceau (Palay, Chan-Palay 1974). Recent morphological studies show that there is variability of the pinceaux size of the basket cell axons on PCs, depending on the module, and this variability is determined by the PC's neurotransmission and maturation (Zhou et al. 2020).

The densely packed granule cell layer is also compartmentalized according to the modular organization (Ozol and Hawkes 1997). However, unlike the input to the PC via the climbing fiber or the more recent findings regarding inhibitory interneurons, the granule cell layer input to PCs, or the mossy fiber input to the granule cell layer does not seem to have strict modular divisions. Climbing fiber bringing in information from the spinal cord follows the modular segregation, but mossy fibers, which bring information from all over the central nervous system, do not respect the modular boundaries (Apps and Hawkes 2009). Climbing fibers from specific olivary neurons terminate in narrow longitudinal bands of 0.2mm to 0.3mm width and this seems to correlate with the width of PC band which fire complex spikes in synchrony (Fukuda, Yamamoto, and Llinás 2001; Sugihara et al. 2007). But, mossy fiber collaterals often distribute themselves bilaterally, way past the narrow longitudinal bands followed by climbing fibers (Sugihara et al. 2001; Voogd et

al. 2003). PCs have been shown to receive input from neighboring microzones, further showing that mossy fiber inputs often disregard the modular boundaries (Valera et al. 2016). Therefore, there is some evidence of possible communication and coordination between modules regarding the mossy fiber input to the cerebellum. But, so far, there is no evidence of communication of modules regarding climbing fiber inputs.

1.3 Cerebellar learning

The cerebellum works as a site for acquisition of motor learning and temporary storage of the memory of the learned motor output (D'Angelo 2018b; Lisberger 2020; Raymond et al. 1996). Two of the main places where motor learning happens are the PCs and the DCN neurons (Lisberger 2020). For this introduction, I will focus more on the PCs. Pavlovian eyelid conditioning, studied mostly in rabbits, is often used to understand how the cerebellum learns and retains memory (D'Angelo 2018b; De Zeeuw 2020; Medina 2000). In its most basic form, a tone which works as a neutral/conditioned stimulus (CS) is followed by a puff to the eyelid which works as an eyelid reflex evoking/ unconditioned stimulus (US) (Ohyama et al. 2003). Sensory information/CS (tone) via the mossy fibers are diverged onto rGCs and then are converged onto PCs via parallel fibers and this provides the cerebellum with contextual information (D'Angelo 2018b; Hesslow, Svensson, and Ivarsson 1999). Climbing fibers from the inferior olive provide the PCs with the error signal/US (air puff on eyelid) and this works as the teaching signal to the cerebellum (Mauk, Steinmetz, and Thompson 1986). After the animal is trained with the

CS and US paired together with specific inter-stimulus intervals between them, it forms a new sensorimotor associative memory such that it can predict the US (air puff) after the CS (tone) accurately and initiate eyelid closure right before the US onset (De Zeeuw 2020; Ohyama et al. 2003).

This is an example of how the cerebellum is involved in predictive feedforward learning, where it combines past experiences and current sensorimotor input to predict the correct response, and it does so by repeated error signal via climbing fiber input to the PCs during its learning phase (Ohyama et al. 2003). The early phase of learning is stored temporarily in the cerebellar cortex by LTD at the parallel fiber-PC synapse which takes place when there is simultaneous climbing fiber input (De Zeeuw 2020). This learned motor memory is transferred, for more permanent storage, to the DCN neurons that receive input from the PCs (De Zeeuw 2020; Heiney et al. 2014).

A specific area in the cerebellar hemisphere responsible for the learned eyeblink response was identified as early as 1994 and it is in lobule VI of the cerebellar hemisphere, also known as HVI (Hesslow 1994). Since then, the region has been shown to be more specific to a microzone on HVI, which is in the deep fissure of HVI and right next to lobule V (Halverson, Khilkevich, and Mauk 2018; Mostofi et al. 2010). This periocular microzone is essential for the learned eyeblink response and receives both mossy fiber and climbing fiber input from the same periocular receptive field (Mostofi et al. 2010).

As described above, the mossy fiber (contextual sensory information) input to the cerebellum is not necessarily restricted to one module because of the architecture of

information flow. But the climbing fiber (error signal) input seems to be restricted to modules for both the OCNO circuit and the output from the cerebellar cortex. So far, there is no known way by which the modular error signal pathways communicate.

Because mGCs are in the molecular layer, they have the potential to receive climbing fiber input directly. In such a scenario, mGCs would be a way for different OCNO circuits of cerebellar modules to communicate via the parallel fibers of mGCs, which can cross modular boundaries.

1.4 Specific Aims

Molecular layer granule cells have been documented in the adult cerebellum of several species such as mice, rats, rabbits, guinea pigs, and humans (Berciano and Lafarga 1988; Brzustowicz 1952; Lafarga and Berciano 1985). And yet, an accurate account of their numbers or function in the molecular layer is unknown. Researchers in the past have deemed them ectopic primarily because they used nonspecific markers and could only identify mGCs in small subpial clusters. It was also speculated that mGCs are the last ones to begin migration and so could not complete their journey to the granule cell layer (Palay, Chan-Palay 1974; Ponti et al. 2008). However, this has not been supported by evidence. After the 1990s, mGCs have not even been mentioned in the context of the normal cerebellum, and as a result their physiological or synaptic properties have not been investigated. Their location in the molecular layer makes them potential recipients of different synaptic inputs compared to those received by rGCs, therefore making mGCs part

of a novel cerebellar circuit. For this dissertation, I have divided the analysis in three major aims, discussed in detail in the three following chapters.

Aim 1. Histochemical characterization of mGCs: I will use antibody staining to check whether mGCs express marker specific for mature granule cells and calculate the fraction of molecular layer cell population that is made up by mGCs. A new transgenic line, TCGO, is now available which marks rGCs and mGCs sparsely. I will use immunohistochemical analysis to verify whether it can be used to identify and study mGCs *in vivo*.

Aim 2. Developmental profile of mGCs: BrdU marks proliferating cells. I will use timed BrdU injections in postnatal animals to check whether mGCs are born within the birth timeline of rGCs, or near the end of the timeline, as was previously speculated. I will use *in vivo* tracking of mGCs over months to investigate if they are slowly migration to the granule cell layer, even in adults, or are stably integrated in the molecular layer.

Aim 3. Physiological characterization of mGCs: Using the TCGO mouse, I will compare the membrane properties of mGCs to rGCs and test whether mGCs can transmit synaptic signal and receive excitatory synaptic input.

The next step after this dissertation is to identify the source of this input. Given the location of mGCs, potential sources are primarily parallel fibers and climbing fibers and infrequent mossy fibers in the molecular layer. A further step would be to use calcium imaging in the intact mouse to check if mGCs are active while provided with a sensory stimulus or taught to perform simple motor tasks.

Chapter 2

Quantification of molecular layer granule cells and validation of transgenic mouse model, TCGO, to identify and study them

Abstract

Molecular layer granule cells (mGCs) are found across species, but their quantity and potential role in the cerebellum remains unexplored. Morphological studies of mGCs have been few and restricted to the visually identifiable clusters labelled by nonspecific staining; mGCs have been reported to be stuck in their immature state. In this chapter, I show that mGCs make up one third of the molecular layer cell population, express marker for mature granule cells, and are distributed throughout the molecular layer. This suggests that earlier studies underestimated the significance of mGCs, a major neuronal group in the molecular layer. A new transgenic mouse line, TCGO, marks granule cells sparsely with the fluorescent protein *mCitrine*. I show that *mCitrine* positive cells in the molecular layer of TCGO mice are exclusively mGCs and so the TCGO mouse line is a valuable tool to study the functional properties of mGCs in slice preparations and *in vivo*.

Introduction

Granule cells have been found in the molecular layer of normal adult cerebellum in various animal species, including mice, rats, rabbits, pigs, and humans (Berciano, Conde, and Lafarga 1990; Berciano and Lafarga 1988; Brzustowicz 1952; Lafarga and Berciano 1985). These studies found clusters of granule like cells near the pia mater and sparsely throughout the molecular layer and called them ectopic granule cells. Berciano and Lafarga reported that ectopic granule cells are predominantly found in the primary fissure, besides

as clusters near the pia mater, and they assumed that it was a result of "small-scale error in migratory behavior" (Berciano and Lafarga 1988).

However, caution is needed before coming to conclusion about mGCs because non-specific histochemical staining techniques, such as hematoxylin-eosin staining and Nissl staining, were used in these studies. With these staining techniques, mGCs are identified unambiguously only when they form a clear, distinguishable cluster near the pia mater. If individual mGCs are widely distributed throughout the molecular layer, they are difficult to identify because their staining pattern appears similar to that of molecular layer interneurons, i.e., stellate cells and basket cells. Therefore, the number of mGCs has likely been underestimated.

Indeed, Golgi staining identified GCs distributed throughout the molecular layer (Lafarga and Berciano 1985; Berciano and Lafarga 1988). Although Golgi staining is non-specific as well, it reveals the entire structure of sparsely labeled cells, allowing the identification of cell-type. The Golgi-stained mGCs often have unbranched and club-shaped dendritic terminals instead of branched and claw-like terminals: the structural characteristics of regular GCs (rGCs) in the granule cell layer. The mean number of mGC dendrites is slightly fewer than that of rGCs (Lafarga and Berciano 1985). Because of their location, mGCs and rGCs may receive different presynaptic inputs and so it is not surprising that they have slightly different dendritic structures. Nevertheless, the structural characteristics of mGCs led to the conclusion that mGCs are stuck in their immature

morphological state. Combined with the assumption that mGCs are a minor population in the molecular layer, they have been dismissed as rare and harmless ectopic neurons.

What has been lacking so far is the precise quantification of mGCs and any functional characterization. Granule cells express $GABA_A$ receptors during postnatal development and the subunit expression pattern changes with maturation stages; granule cell precursors express the subunits: α2, α3, β3, γ1 and γ2 whereas expression of the subunit α6 is restricted to post migratory mature granule cells (Laurie, Wisden, and Seeburg 1992). $GABA_A$ receptor α6 ($GABA_AR$α6) subunit is now used as an established marker for mature granule cells in the granule cell layer (Kato 1990; Kim et al. 2014; Mellor et al. 1998). Furthermore, transgenic animals and virus-mediated gene delivery systems are now widely used to label specific cell populations with fluorescent proteins for their functional characterization in live specimens.

To reevaluate mGCs with these new tools, I first quantified the fraction of mGCs, i.e., $GABA_A$ receptor α6-positive cells, in the entire population of molecular layer cells in normal adult mice. I found that mGCs are one of the most abundant cells in the molecular layer and make up about one third of the cell population of the molecular layer. My result suggests that mGCs are not negligible and erroneous neurons as previous studies assumed. I also found that mGCs are selectively labeled by the fluorescent protein *mCitrine* in the previously reported transgenic mouse line, called TCGO (Huang et al., 2013; Shima et al., 2016). I show that no cells other than mGCs are labeled in the molecular layer of the TCGO

mice. Therefore, TCGO mice can be used as an experimental tool for further analysis of mGCs, including their physiological properties and migratory capability.

Materials and Methods

Animals

C57BL/6J (B6) mice were used for quantification of the fraction of mGCs in the molecular layer. Adult TCGO mice were used to validate that *mCitrine* positive cells in the molecular layer are mGCs which express markers for mature granule cells. All mice used for this dissertation were four months of age or older, unless otherwise stated. All mice procedures were performed in accordance The Institutional Animal Care and Use Committee (IACUC) and were approved by the University of Texas at Austin Institutional Animal Resource Center.

Immunohistochemistry and quantification

Mice were anaesthetized with an intraperitoneal injection of ketamine/xylazine (100/10 mg/kg) and then intracardially perfused with 4% paraformaldehyde in phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4 , pH 7.4). The cerebellums were extracted post-perfusion and further fixed overnight at 4 °C by immersion fixation in 4% paraformaldehyde in 1xPBS. The next morning, they were washed in fresh 1xPBS at room temperature 4 to 5 times at 30-minute intervals. Sagittal

sections of the cerebellum were made at 60 µm thickness and immunostained for $GABA_AR\alpha6$ and GAD67 separately. The sections were washed 3 times at 5-minute intervals in 0.5% Triton X-100 in PBS (PBST) on a shaker and at room temperature.

To label mGCs or interneurons in B6 mice, sections were blocked in 5% normal donkey serum (NDS) in PBST for 1 hour at room temperature. The immunohistochemistry (IHC) protocol followed was similar for both $GABA_AR\alpha6$ and GAD67. Sections were incubated overnight at 4 °C on a shaker with anti- $GABA_AR\alpha6$ antibody (1:500 diluted in 5% NDS in PBST, Synaptic Systems-224 603). For GAD67, recombinant Anti-GAD67 antibody (1:500 diluted in 5% NDS in PBST, Abcam ab-213508) was used. They were washed 3 times at 5-minute intervals in PBST on a shaker and at room temperature. Next, they were incubated with secondary antibody separately for $GABA_AR\alpha6$ and GAD67 for 2 hours at room temperature. For both, the same secondary antibody was used; donkey anti-rabbit IgG H&L Alexa Fluor 568 (1:500 diluted in 5% NDS in PBST, Abcam-ab175470). The sections were then washed 3 times at 5-minute intervals in PBST and mounted with mounting media containing DAPI (DAPI Fluoromount-G, SouthernBiotech). Sections were coverslipped and imaged using a laser-scanning confocal microscope (FV-1000, Olympus, Tokyo, Japan). A 40x water immersion objective lens (0.8 N.A.) was used. Z-stack images of the entire depth of the molecular layer was acquired at 1.5 µm step size using the 405 nm and 543 nm lasers, sequentially. The emitted blue and orange fluorescent signals were separated by a dichroic mirror (a long pass filter at 490 nm) and then filtered by emission filters (bandpass filters 430–470 and 560–660 nm for blue

and orange emissions, respectively). The $GABA_AR\alpha6$ immunostaining was also performed with the cerebellum obtained from an adult Sprague-Dawley rat.

For quantification, the total number of DAPI positive cells in each imaged section was counted and then the number of the DAPI positive cells that were also positive for either $GABA_AR\alpha6$ or GAD67 were counted. The fraction of $GABA_AR\alpha6$ positive cells and GAD67 positive cells in the molecular layer was reported. For each set, data from 4 B6 mice were used. The field of view was selected randomly from the entire cerebellum. The quantification was done across multiple z-sections in the same field of view and the volume range for quantification was from $0.0013mm^3$ to $0.0116mm^3$. The average fraction and standard deviation were reported for each group.

To label mGCs, interneurons, or NG2+ glial cells in TCGO mice, the above primary antibodies and anti-NG2 rabbit polyclonal antibody (1:500, AB5320, Millipore Sigma) were used. Donkey anti-rabbit IgG H&L Alexa Fluor 647 (1:500 ab150075, Abcam) was used as the secondary antibody for double-color detection with *mCitrine*.

For all three IHCs ($GABA_AR\alpha6$, GAD67 and NG2) at least 4 TCGO mice were used to thoroughly examine cerebellar sections.

Results

Molecular layer granule cells (mGCs) have been thought to be a small number of ectopic neurons stuck in their immature state, clustered near the pia mater. These conclusions were drawn more than several decades ago using nonspecific, and sometimes

sporadic staining methods, such as hematoxylin-eosin staining and the Golgi staining; hence, the distribution and abundance of mGCs remain unclear (Berciano and Lafarga 1988; Lafarga and Berciano 1985). But now, $GABA_AR\alpha6$ is widely used as an established marker for mature granule cells. As the first step for reevaluating mGCs, I have conducted IHC on B6 mice using the $GABA_AR\alpha6$ antibody to examine the accurate distribution and abundance of mGCs in the normal adult cerebellum.

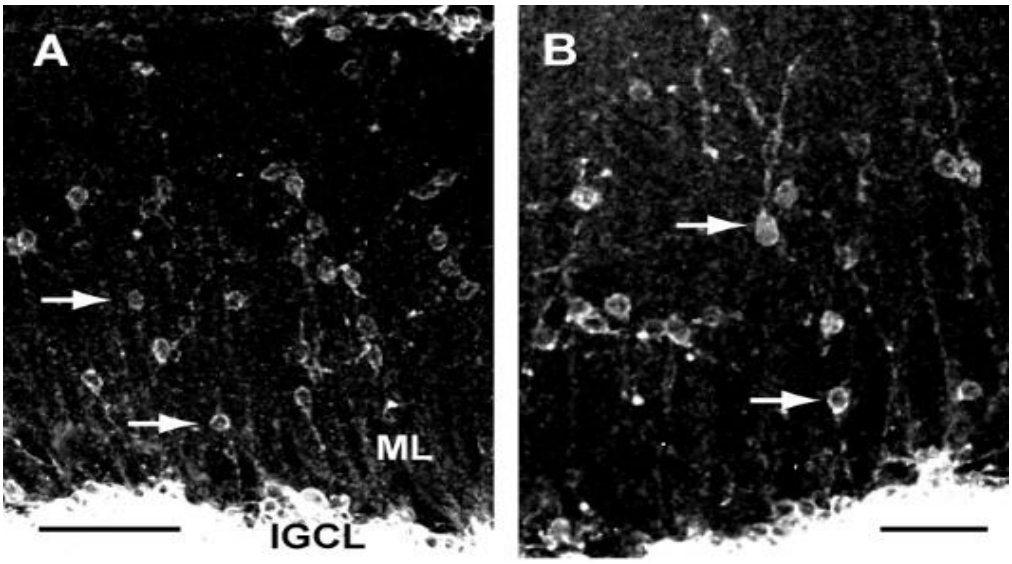

Figure 4.

mGCs are distributed throughout the molecular layer in both mice and rats.

4**A**. Representative z-stack image of IHC for $GABA_AR\alpha6$ (white) on molecular layer of B6 mouse cerebellum, showing that mGCs are distributed evenly throughout the molecular layer. 4**B**. Representative z-stack of IHC for $GABA_AR\alpha6$ (white) on rat cerebellum, showing even distribution of mGCs in the molecular layer. White arrows in both images are pointing to individual mGCs with $GABA_AR\alpha6$ expression on the membrane showing that they are mature granule cells. The top is the pial surface and the bottom is the internal granule cell layer. Scale bar is 30 μm.

Molecular layer granule cells are distributed throughout the molecular layer at any depth from the pia mater (Fig. 4A). This distribution pattern appears similar across the cerebellar lobules and is observed in adult rat cerebellum as well, indicating that the wide distribution of mGCs is not restricted to mice (Fig. 4B).

The adult cerebellar molecular layer is known to consist of primarily inhibitory interneurons and some NG2 glial cells. Molecular layer granule cells are not mentioned in the current cerebellar literature as a part of the cellular makeup of the molecular layer (D'Angelo 2014, 2018a; Ruigrok et al. 2015). I have used IHC on B6 mice using $GABA_AR\alpha6$, GAD67, and DAPI to calculate the proportions of mGCs and inhibitory interneurons that make up the adult molecular layer. DAPI, 4′,6-diamidino-2-phenylindole, binds to the adenine-thymine rich regions of the DNA and fluoresces in the blue range. Therefore, it is used to count the total number of cells. GAD67, or glutamic acid decarboxylase marks all GABAergic neurons, or inhibitory interneurons (Uusisaari and Knöpfel 2011). Therefore, GAD67 was used as a marker for both basket and stellate cells. The total number of cells I counted in the molecular layer from the 4 animals is 5,090. Among these, the total number of cells that are also positive for GAD67 is 2,708. Therefore, the average fraction of GAD67 positive cells per animal is 0.558 and the standard deviation is ± .039 (Fig. 5C, right bar). My data shows that interneurons do not account for the entire cell population of the molecular layer cells but rather form only about two thirds of the molecular layer cell population.

The ubiquitous presence of mGCs in the molecular layer was already recognized from my IHC data. I quantified the fraction of mGCs which make up the molecular layer. As described above, $GABA_A\alpha6$ was used as a marker for mGCs. The total number of molecular layer cells I counted from 4 animals is 6,531 and among those, the total number of cells that are also positive for $GABA_AR\alpha6$ is 1,956. Therefore, the average fraction of molecular layer cells that is $GABA_AR\alpha6$ positive (per animal) is 0.297 and the standard deviation is ± 0.022 (Fig. 5C, left bar). My data shows that mGCs are not just few erroneous neurons, but rather, make up one third of the molecular layer population. GAD67 marks both basket and stellate cells, and combined, they make up two thirds of the molecular cell population. These findings suggest that mGCs may be the most abundant cell type of the molecular layer.

Identifying mGCs is difficult, as exemplified by the underestimation of their numbers in the past, and it is even more so in live tissue. But a mouse line was reported by Huang *et al*., which labels rGCs sparsely with a fluorescent protein (*mCitrine*), and potentially also labels mGCs. Random insertion of enhancer-trap lenti-viral vectors via mouse embryonic infection was used to generate multiple mouse lines which label small subset of neurons; one of the lines is called TCGO (Shima et al. 2016). The TCGO mouse expresses the fluorescent protein *mCitrine* randomly and sparsely in granule cells of the granule cell layer (rGCs) (Huang et al. 2013). By quick screening of cerebellar sections of the TCGO mouse, I found that *mCitrine* is also expressed in mGCs. This is a potentially good tool to identify mGCs in live tissue.

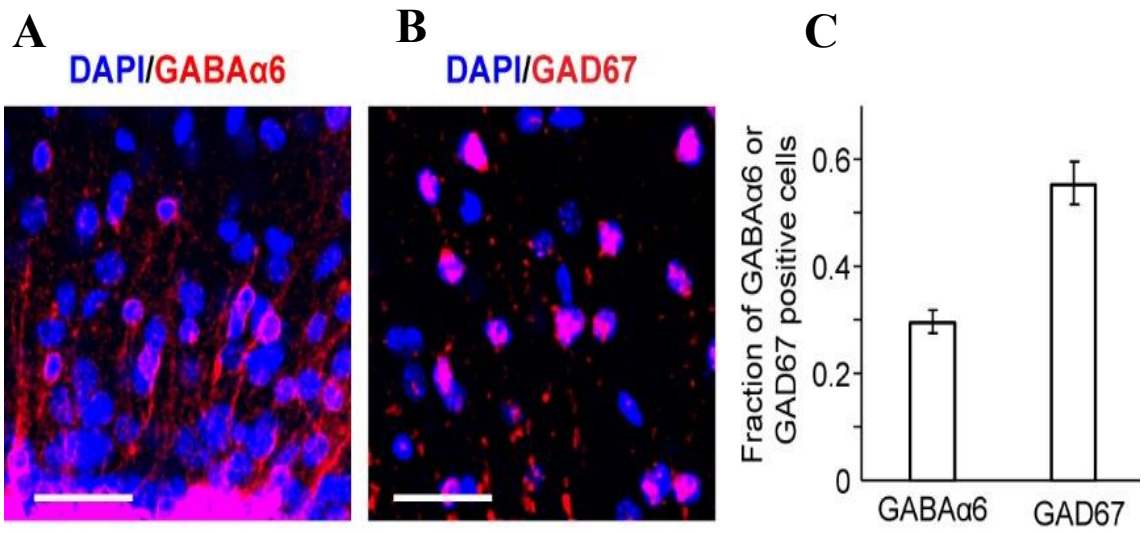

Figure 5.

mGCs are one of the most abundant neurons in the molecular layer.

A. Representative merged z-stack image of DAPI (blue) and $GABA_AR\alpha6$ (red) IHC signals showing mGCs, which express $GABA_AR\alpha6$, are one of the most abundant neurons of the molecular layer. They are evenly distributed throughout the molecular layer. **B**. Representative z-stack image of DAPI (blue) and GAD67 (red) IHC signals showing that there are a significant number of DAPI positive cells which do not overlap with GAD67, showing that not all/most molecular layer cells are interneurons, as previously thought.
C. Bar graphs showing the fraction of cells in the molecular layer that are mGCs (left), 0.297, and fraction of cells which are inhibitory interneurons, 0.558. Error bars show the standard deviation. Scale bar is 30 µm.

But, before using them to study mGCs, I needed to validate that *mCitrine* is only expressed in mGCs which express $GABA_AR\alpha6$ and not in any other cell type found in the molecular layer, which are mostly inhibitory interneurons interspersed with few NG2+ glial cells. I verified whether the *mCitrine* expression met three key conditions: **a**. *mCitrine* expression should overlap with $GABA_AR\alpha6$ expression, **b**. *mCitrine* expression should not overlap with GAD67 expression, and **c**. *mCitrine* expression should not overlap with NG2

expression. Figure 6 is a visual representation of the three conditions that needed to be met before the TCGO mouse could be used for studying mGCs.

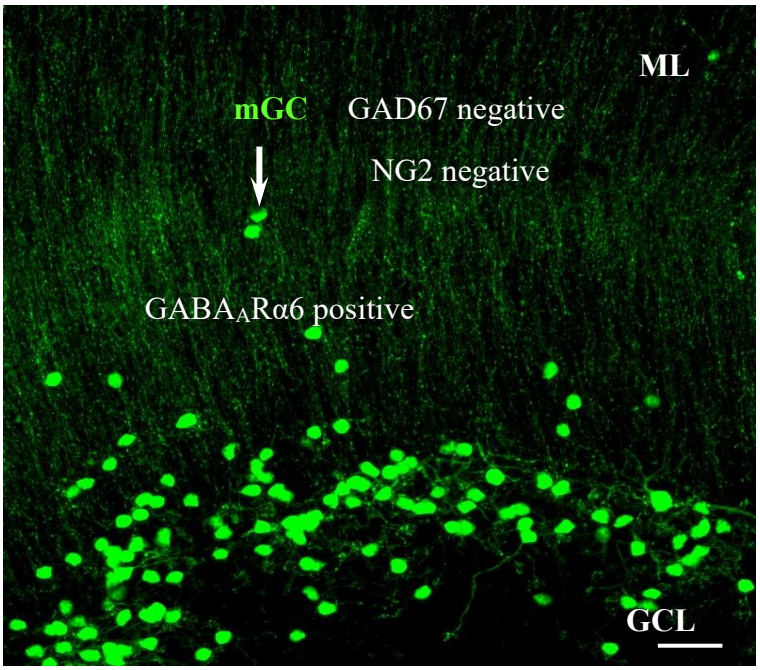

Figure 6.

TCGO mice validation.

Sagittal section of TCGO cerebellum, pial surface is on top. Sparse *mCitrine* expression is in granule cells of the granule cell layer and molecular layer. White arrow is pointing to mGCs expressing *mCitrine*. Validation of TCGO mouse line: the conditions listed in a., b., and c. need to be true for all *mCitrine* positive cells of the molecular layer. Scale bar is 20 µm.

All TCGO molecular layer sections with IHC carried out separately for GABA$_A$Rα6, GAD67, and NG2 antibodies were thoroughly examined to check for the three conditions listed above. In all sections examined, the three conditions listed were met (Fig 7). None of the *mCitrine* positive mGCs co-express either GAD67 or NG2 (Fig. 7B', C') showing that none of the *mCitrine* positive cells in the molecular layer are inhibitory interneurons or NG2+ glial cells. All mGCs which express *mCitrine* in the TCGO mouse also express GABA$_A$Rα6 (Fig. 7A'), showing that they are mature granule cells. Therefore, the TCGO mouse can be used to study mGCs.

There were more cells in the molecular layer which were positive for $GABA_AR\alpha6$ but did not express *mCitrine* and this is not surprising because *mCitrine* labels granule cells sparsely, as is also observed for rGCs.

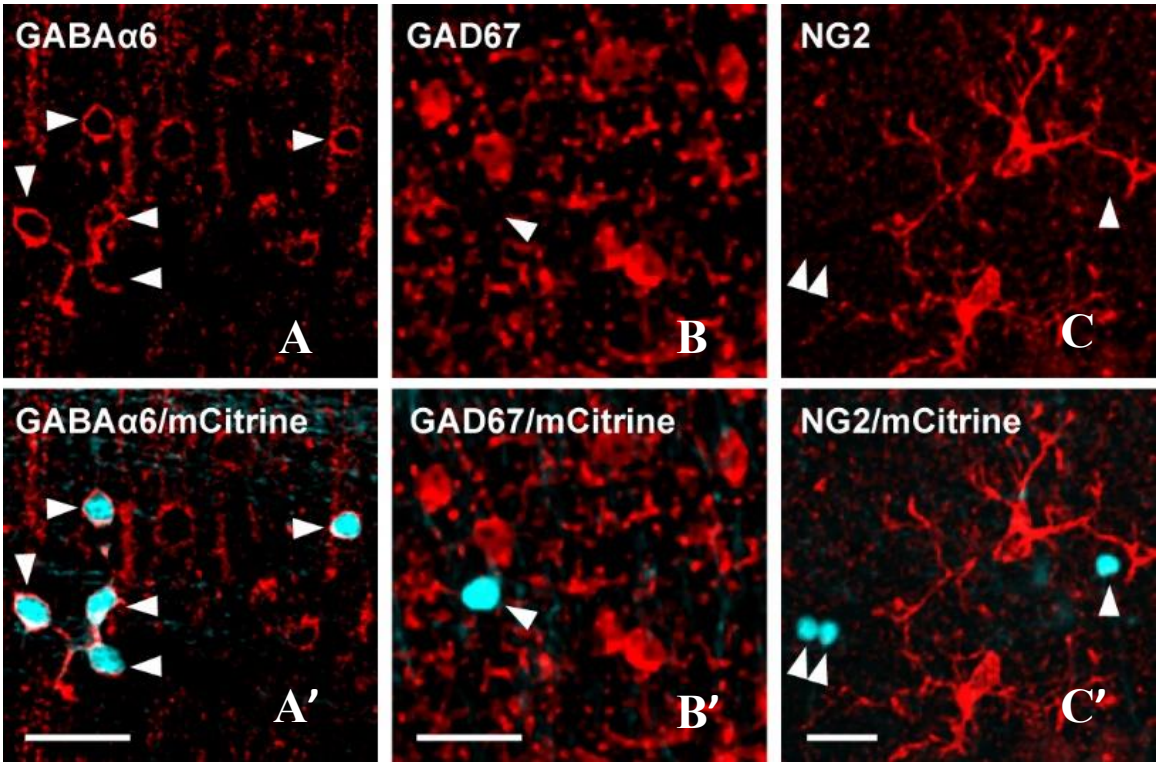

Figure 7.

TCGO mice express *mCitrine* exclusively in mGCs in the cerebellar molecular layer.

The top panels (**A**, **B**, and **C**) show the molecular layer of the TCGO mice immunostained for $GABA_AR\alpha6$, GAD67, and NG2 in red. The bottom panels (**A'**, **B'**, and **C'**) show the same panels with the 3 immunostains along with the *mCitrine* expression. In 7 **A** and **A'**, the arrowheads are pointing to the *mCitrine* expressing mGCs which also express $GABA_AR\alpha6$. In 7 **B**, **B'**, **C**, **C'**, the arrowheads point out that *mCitrine* expressing mGCs do not overlap with GAD67 or NG2. Scale bar is 20 µm.

Discussion

Using specific marker for mature granule cells I show that mGCs are not restricted to ectopic clusters near the pia mater but are distributed throughout the adult cerebellum. Moreover, they express $GABA_AR\alpha6$, which is a marker for mature granule cells. This indicates that even though some mGCs may seem to be in a morphologically immature stage, as noted by Lafarga and Berciano, they potentially have membrane properties identical or similar to mature granule cells.

The neuronal makeup of the molecular layer has been thought to be mostly inhibitory interneurons and they have been the focus of the molecular layer circuitry. Because mGCs were deemed ectopic, their synaptic or functional properties have not been studied so far. I show that mGCs make up one third of the normal adult molecular layer and maybe the most numerous cell type of the molecular layer. Unlike rGCs which are in the granule cell layer, mGCs are in the molecular layer; the molecular layer has a different set of dendrites and axons compared to those which are encountered by the rGCs. Therefore, it is possible that mGCs have different synaptic connectivity and make a novel circuitry in the cerebellar cortex.

It is difficult to identify mGCs in live tissue, but a new transgenic mouse line, TCGO, is a potential solution to this problem. In the TCGO mice line, rGCs are labelled sparsely with *mCitrine* (Huang et al. 2013; Shima et al. 2016). I used IHC to validate that the TCGO line also labels only mGCs with *mCitrine*, thus showing that this new tool may be used to study the functional properties of mGCs in slice preparations or *in vivo*. Using

the TCGO mouse, we can now begin to investigate the role of mGCs in the cerebellum. I have used the TCGO mouse line for the remainder of my dissertation to study the birth timing, migration, physiological, and synaptic properties of mGCs.

Chapter 3

Developmental profile of molecular layer granule cells (mGCs)

Abstract

Since the discovery of mGCs, they a have been considered ectopic neurons which were stuck in the molecular layer, *en route* to the granule cell layer. The existing hypothesis is that mGCs are a result of the very last granule cell precursors to differentiate and the signaling mechanism for neural migration stopped before they could reach the granule cell layer. A more recent study suggests that some mGCs might be migrating to the granule cell layer, even in adult animals. I used cell division markers during different postnatal days and tracked them in adult mice to test whether mGCs are born last. Quantification of the fraction of mGCs versus rGCs born at three different postnatal days suggest that mGCs are born within the same time frame as rGCs, a few days delayed, but not at the end. I use two photon imaging *in vivo* over the course of several months to test whether mGCs migrate to the granule cell layer even in adults. My data suggest that mGCs are not motile and this indicates that mGCs are stably integrated in the molecular layer circuitry.

Introduction

The external granule cell layer is a proliferative zone on the cerebellar surface and it is the source of all granule cells (Goldowitz 1998; Daniver Morales and Hatten 2006). The proliferative zone gives rise to the granule cell precursors which undergo proliferation alone until around birth, they start differentiating around P3, and migrate towards the nascent granule cell layer (Espinosa and Luo 2008). The external granule cell layer persists until postnatal week 3; but continues to become sparser with time, eventually disappearing

around postnatal day postnatal day 18-21 (P18 to P21) (Espinosa and Luo 2008; Rahimi-Balaei et al. 2018). The granule cell layer becomes discernible around P5 and continues to grow until around P21, when the granule cell layer is fully formed (Espinosa and Luo 2008).

The granule cell precursors divide symmetrically throughout the embryonic and postnatal stages of the mouse but they do not divide at the same rate throughout this time (Espinosa and Luo 2008; Fujita 1967). Before P4, the granule cell precursors are primarily expanding, not differentiating, at a fast rate of one mitotic division per day; from about P5 to P6 the proliferation rate goes down to once every two days and continues to decline with time until the entire precursor population is exhausted around P18 (Espinosa and Luo 2008; Fujita 1967). From around P5 to P15, the precursors undergo a mix of proliferation and differentiation, with the top most layers of the external granule cell layer dividing and the lower ones differentiating; differentiation of the precursors predominates after P15 (Espinosa and Luo 2008; Fujita 1967). Each time, the granule cell precursors undergo small burst in proliferation just before they differentiate (Espinosa and Luo 2008). The differentiating granule cells extend their axons in both directions in the molecular layer and descend down to the granule cell layer, guided by the Bergmann glial processes, and this transit through the molecular layer takes 28 to 31 hours (Fujita 1967; Rahimi-Balaei et al. 2018). Granule cells stack their axons in the molecular layer in a chronological order; the earlier differentiating ones stack their axons closer to the granule cell layer and later

differentiating ones stack them closer to the pia mater (Espinosa and Luo 2008; Zong et al. 2005).

It has been hypothesized that mGCs result from the very last granule cell precursors to differentiate which could not complete their transit to the granule cell layer (Berciano et al. 1990). The reasoning for the hypothesis was that because mGCs need to cross a more complex molecular layer and more parallel fibers obstructing their migratory path, they cannot reach the granule cell layer before the intrinsic and extrinsic migratory cues stop (Berciano et al. 1990).

After the 1990s, mGCs in the normal adult cerebellum have been mostly ignored, but one study on adolescent rabbits suggested that some mGCs might still be migrating late towards the granule cell layer (Ponti et al. 2008). Around the 4^{th} to 5^{th} postnatal week, the external granule cell layer is replaced by a proliferative subpial layer which mostly produces interneurons and synaptocytes (Ponti et al. 2008). Even as late as the 3^{rd} to 4^{th} postnatal months, some bipolar shaped migratory granule cells were observed in the molecular layer but do not become mature granule cells because they do not express the marker, which is $GABA_A$ receptor subunitα6 (Ponti et al. 2008).

The mouse does not have the secondary proliferative subpial layer and, to my knowledge, at least a significant portion of mGCs express $GABA_AR\alpha6$. However, considering that some granule cells migrate in rabbits as late as 4 months and the prior assumptions about mGCs being the last ones to be born, I tested two possibilities: 1.) mGCs

in mice are the last ones to go from proliferation to differentiation, and 2.) mGCs are migrating late and slowly towards the granule cell layer, even in adults.

I used BrdU injection as timed proliferation marker and two-photon *in vivo* imaging to track mGCs using the TCGO mouse (introduced in chapter 2) to test both possibilities. I found that mGCs are not a result of the very last granule cell precursors to differentiate. The precursors which become mGCs are mostly formed about a few days after the peak of all granule cell precursor proliferation, but still within the timeframe of normal granule cell birth. Time-lapse *in vivo* imaging data show that mGCs are not migrating late towards the granule cell layer in adult mouse, which suggest that they are stably situated in their location and integrated into the molecular layer circuitry.

Material and Methods

Animals

C57BL/6J (B6) mice were used for the finding the birth time frame of mGCs. B6 pups at different postnatal ages were used for BrdU injection and housed separately for 1 week post injection. TCGO mice starting at 1 month of age were used to track mGCs *in vivo*. All mice procedures were performed in accordance The Institutional Animal Care and Use Committee (IACUC) and were approved by the University of Texas at Austin Institutional Animal Resource Center.

BrdU injection

BrdU was dissolved in sterile phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4), prewarmed in a water bath at around 45°C, at the maximum solubility of 20 mg/ml via sonication and periodically vortexing between sonication cycles. B6 pups at either P4, P8, or P12 were subcutaneously injected with BrdU solution at 150 mg/kg. Two pulses of BrdU were injected at 4 hour interval for each pup at for the selected timepoints. Pups were housed along with their mother in a secluded cage (per mother-litter set) for one week after injection and then in regular cages. Pups were allowed to survive for at least 8 weeks post BrdU injection. They were then perfused, and their cerebellums were processed for immunohistochemistry.

Immunohistochemistry and quantification

BrdU injected animals were anaesthetized with an intraperitoneal injection of ketamine/xylazine (100/10) and then intracardially perfused with 4% paraformaldehyde in phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4). The cerebellums were extracted post-perfusion and further fixed overnight at 4 °C by immersion fixation in 4% paraformaldehyde in PBS. The next morning, they were washed in fresh PBS at room temperature 4 to 5 times at 30-minute intervals. Sagittal sections of the cerebellum were made at 60 µm thickness and immunostained for BrdU and $GABA_AR\alpha6$ together. The sections were washed 1x overnight at 4°C on a shaker in 0.5% Triton X-100 in PBS (PBST). The following morning,

antigen retrieval was carried out by keeping them submerged in 2N HCl at room temperature on a shaker for 30 minutes. The 2N HCl was rinsed off thoroughly by washing them 2 times at 10-minute intervals in PBST on a shaker and at room temperature. They were blocked in 5% normal donkey serum (NDS) in PBST for 1 hour at room temperature. Sections were incubated overnight at 4 °C on a shaker with anti- $GABA_AR\alpha6$ antibody (1:500 diluted in 5% NDS in PBST, Synaptic Systems- 224 603) and anti- BrdU antibody (1:500 diluted in 5% NDS in PBST, Abcam- ab6326), together.

Next morning, they were washed 3 times at 5-minute intervals in PBST on a shaker and at room temperature. Next, they were incubated with secondary antibody together for both $GABA_AR\alpha6$ and BrdU for 2 hours, at room temperature, on a shaker, and protected from light. For $GABA_AR\alpha6$, the secondary antibody used was donkey anti-rabbit IgG H&L Alexa Fluor 488 (1:500 diluted in 5% NDS in PBST, Abcam-ab150073). For BrdU, the secondary antibody used was donkey anti-rat IgG H&L Alexa Fluor 568 (1:500, ab175475, Abcam). The sections were then washed 3 times at 5-minute intervals in PBST and mounted with mounting media (Permafluor, Thermo Fisher Scientific, Waltham, MA.). Sections were coverslipped and imaged using a laser-scanning confocal microscope (FV-1000, Olympus, Tokyo, Japan). A 40x water immersion objective lens (0.8 N.A.) was used. Z-stack images of the entire depth of the molecular layer was acquired at 1.5 µm step size using the 488 nm and 543 nm excitation lasers. The green ($GABA_AR\alpha6$) and orange (BrdU) fluorescent emissions were separated by a long-pass filter 560 nm and then filtered by bandpass filters 505–525 nm and 560–660 nm, respectively.

I calculated the fraction of mGCs and rGCs which underwent proliferation and (differentiation soon after) at P4, P8, or P12. The calculation for mGCs was done from cell counts in the molecular layer and the calculation for rGCs was done from cell counts in the granule cell layer. For either layer, the total number of $GABA_AR\alpha6$ positive cells in each imaged section was counted and then the number of the $GABA_AR\alpha6$ positive cells that were also positive for BrdU were counted. BrdU and $GABA_AR\alpha6$ co-labelled cells were the ones born at the particular time points selected (P4, P8, and P12). For postnatal day BrdU injection data set, data from at least 4 B6 mice were used for each injection timepoint. The field of view was selected randomly from the entire cerebellum. The quantification was done across multiple z-sections in the same field of view and the average volume for quantification was $0.0014mm^3$. The average fraction and standard deviation were reported for each group.

Cerebellar window surgery and imaging

Adult TCGO mice were anesthetized with isoflurane. They were first exposed to 4-5% isoflurane in an induction chamber and were transferred on to a stereotaxic device (Stoelting) where the anesthesia was maintained by 1-2% isoflurane at a flow rate of 0.5-1 liters/minute via a nose cone. The mouse's head was secured in position, hair above head was shaved off, eyes were covered with ophthalmic ointment, heat support was provided by placing microwavable heating pads covered with gauze. After the shaved head surface was cleaned with betadine solution, the scalp and neck muscles over the cerebellar region

was removed to expose the cranium. Then the smaller muscles and fascia over the cranium were removed under a surgical microscope and the skull surface was cleaned and dried with a sterile cotton applicator. Surgical cyanoacrylate (Vetabond, 3M) was used to attach a small zinc plate to the skull near lambda, on the parietal bone, perpendicular to the sagittal suture, and extended out to one side (right) for head fixing the animal to perform anesthetized imaging. The zinc plate used was 12x2 mm in length and ~0.9mm in thickness; it was permanently glued in place with dental cement (Ortho-Jet, Lang Dental). The animal's head was held securely by clamping the metal plate to a clamp and surgical cyanoacrylate was used to stop muscles from bleeding. A rectangular craniotomy (~2 × 1.5 mm) was performed using 18 gauge needle tip followed by a high-speed dental drill (#18000–17, Fine Science Tools) with a small drill bit (tip diameter is 0.5 mm, #19007–05, Fine Science Tools) attachment. The dura was left intact and a slightly smaller glass window was put on the exposed brain surface in place of the bone that was removed. The window was cut from a No. 1 coverslip with a diamond scriber (#52865-005, Andwin Scientific). Once the brains surface was clean of blood and bleeding had stopped (if needed) the glass window was secured in place using surgical cyanoacrylate very sparsely to keep the window clear for 2-Photon time-lapse imaging. Dental cement was applied around the glass window and the exposed skull surface, extending to any exposed area. The animal was allowed to recover in single cages and were aided with an analgesic, carprofen (5 mg/kg), administrated daily for 2 days post-surgery.

The animals were allowed to recover for a week and then were imaged and continued to be imaged if the window was clear of blood. The three dimensional region of interest for imaging was kept constant for each animal by using the vasculature on top, and the first layer of rGC layout at the bottom of the z-plane, as landmarks. Anesthesia was induced and maintained by isoflurane as described above and the animals head was kept in position by clamping the metal plate to the microscope stage. Two-photon images were taken using a laser scanning confocal microscope (FV1000MPE, Olympus). Images of *mCitrine* positive cells (granule cells) were taken at 920 nm wavelength provided by Mai Tai HP DeepSee mode-locked Ti: sapphire laser (Spectra-Physics) with a water-immersion ×25 objective lens (1.05 NA), with 1.5 µm step size and at 1x and 3x magnifications. Before final images of regions of interest for each animal were taken, the animal was adjusted using the landmarks as guide for optimal head positioning.

Animals were imaged at one week intervals for as long as possible depending on window clarity and overall animal health. For quantification, the *mCitrine* positive cells in the molecular layer of the 3x magnifications were used and the corresponding 1x magnification images were used as references if needed. For each animal, the number of cells and their 3D location (or any change) was noted, across all timepoints. TCGO animals express *mCitrine* in the parallel fibers (in the molecular layer) as well and these were occasionally used as reference points for exact location to avoid any ambiguity of mGC position.

The data set had a total of 344 mGCs from 17 mice. I narrowed down the number of mGCs tracked for at least a month and this left the data set with 181 mGCs from 10 animals. They were further sorted by the age at which the animal was first imaged to check whether age of animal had any effect on mGC migration, or lack thereof.

Results

The external granule cell layer proliferates rapidly and then slows down with postnatal age (Espinosa and Luo 2008). The rate of precursor proliferation resulting in rGCs is highest from P1 to P5, continues to increase more moderately from P6 to P10, and rate declines from P11 to almost none by P15/18 (Fujita 1967). I have broken down the proliferation in three phases by the speed, as shown by the colored bars superimposed on the graph (Fig 8). I selected P4, P8, and P12 as timepoints to represent the phases because the fraction of proliferating rGCs is almost 100% at P4, about 50% at P8, and 10-20% at P12.

The thymidine analog 5-bromo-2'-deoxyuridine (BrdU) is taken up into the DNA of diving cells. To check the timing of proliferation to differentiation of mGCs versus rGCs, I injected BrdU at the selected time points and sacrificed the mice after two months (Fig 9A). BrdU is passed on to daughter cells and so it dilutes with generations and granule cell precursors undergo a burst of proliferation before terminal differentiation (Espinosa and Luo 2008). Therefore, the cells which I could identify as BrdU positive were derived from the precursors that differentiated within a small time frame of BrdU injection.

The cerebellar sections were double immunostained with anti-BrdU and anti-$GABA_AR\alpha6$ antibodies (Fig.9A). For the molecular layer and granule cell layer, I separately quantified fraction of BrdU-positive cells among the $GABA_AR\alpha6$-positive cells; this gave the fraction of mGCs versus rGCs born at P4, P8, and P12 (Fig. 9A). The rGC group was used as control.

The average fraction of BrdU labelled rGCs when BrdU was injected at P4 is the highest (0.506 ±0.066, n=4 mice), decreases at P8 to about half (0.305±0.095, n=5 mice), and is lowest at P12 (0.121±0.057, n=4 mice) (Fig. 9C). These control numbers agree with the general trend of decreasing proliferation rate.

The average fraction of BrdU labelled mGCs when injected with BrdU at P4 is relatively low (0.14 ±0.019, n=4 mice), is highest at P8 (0.364±0.021, n=5 mice), and low again at P12 (0.177±0.028, n=5 mice) (Fig. 9C'). This data suggests that mGCs are born within the same timeframe as rGCs, but mGCs production peaks around P8. The significant decrease of BrdU-positive mGCs from P8 to P12 injection ($P = 0.007$, ANOVA and post-hoc Tukey HSD test) suggests that mGCs are not merely the last group of granule cells that exit the mitotic cycle.

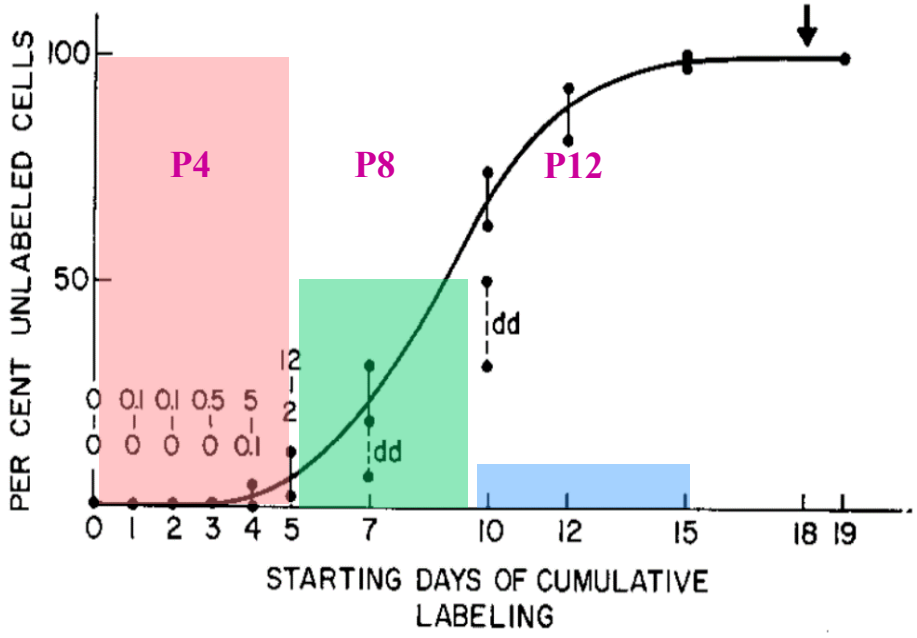

Figure 8.

Percentage of rGCs unlabeled at P20 after H3-thymidine injection from P0 to P19.

Average percentage of H^3-thymidine unlabeled rGCs counted at postnatal day 20. The x-axis shows postnatal days at which H^3-thymidine was injected. From P1 to P10, the upper values represent counts in the vermal region, and the lower values represent those in the hemisphere. Arrow at age of 18 days indicates the time of disappearance of the external granular layer in the Purdue mouse. The three colored boxes represent my breakdown to the fast, moderate, and slow phases of proliferation with the representative timepoints I used indicated in bold fuchsia. Adapted from Fujita, 1967.

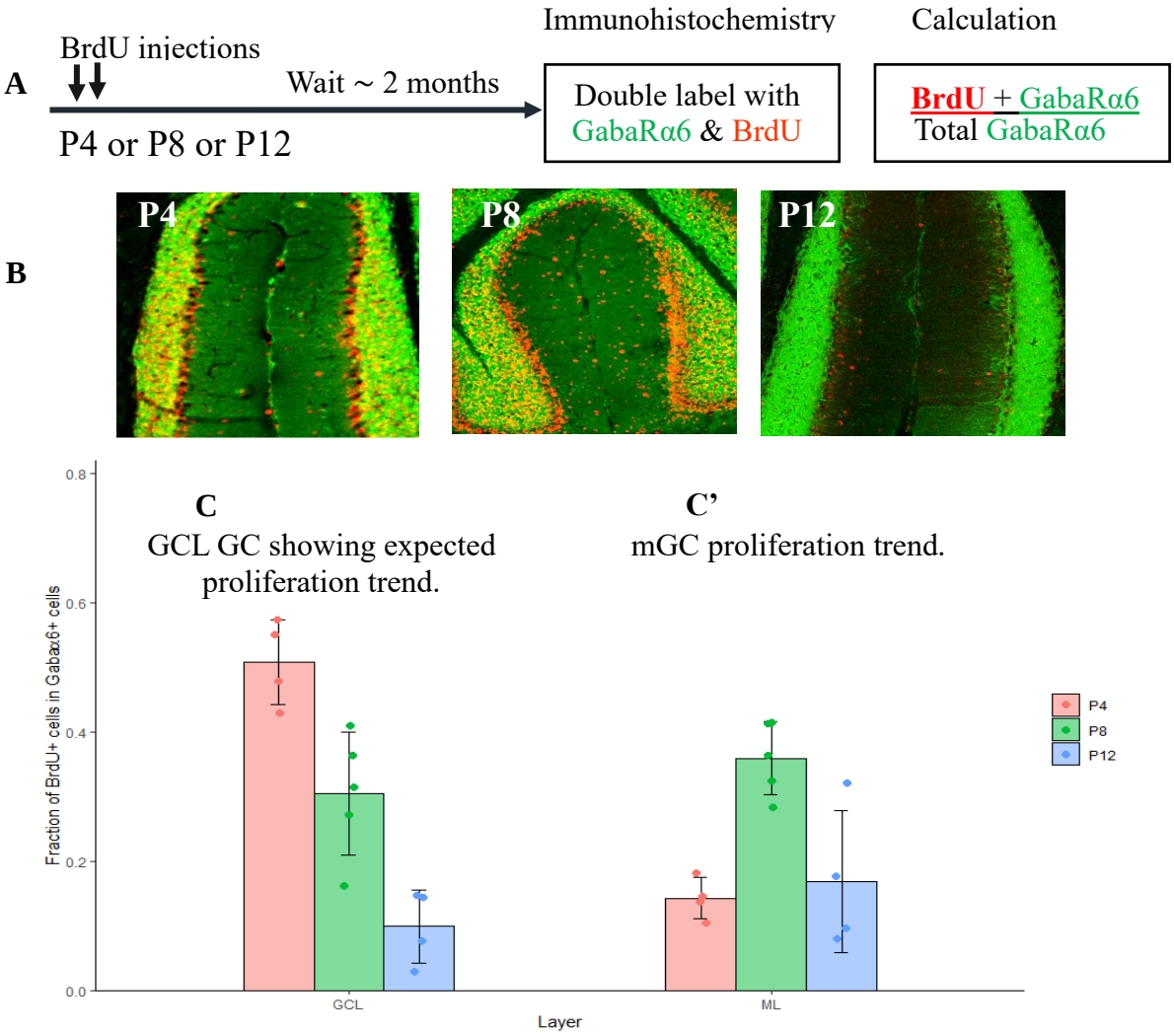

Figure 9.

mGCs are born within the same timeframe as rGCs, but the peak time is shifted.

A. Schematic of experimental set up to check peak birth timeframe of mGCs vs. rGCs.
B. Representative images of cerebellar sections double immunostained with anti-BrdU (red) and anti $GABA_AR\alpha6$ (green) antibodies after 2 months post BrdU injection at P4, P8, or P12. The images show the entire folia with the granule cell layer surrounding the molecular layer. BrdU (red) density in granule cell layer is highest when mice were injected at P4, moderate when injected at P8, and lowest when injected at P12. In the molecular layer, BrdU (red) density is highest when mice were injected at P8, low when injected at either P4 or P12. C&C' are quantification of the fraction of rGCs (**C**) and mGCs (**C'**) that are born at P4, P8, or P12. Peak birth time for rGCs is P4 and P8 for mGCs. Error bars show standard deviation.

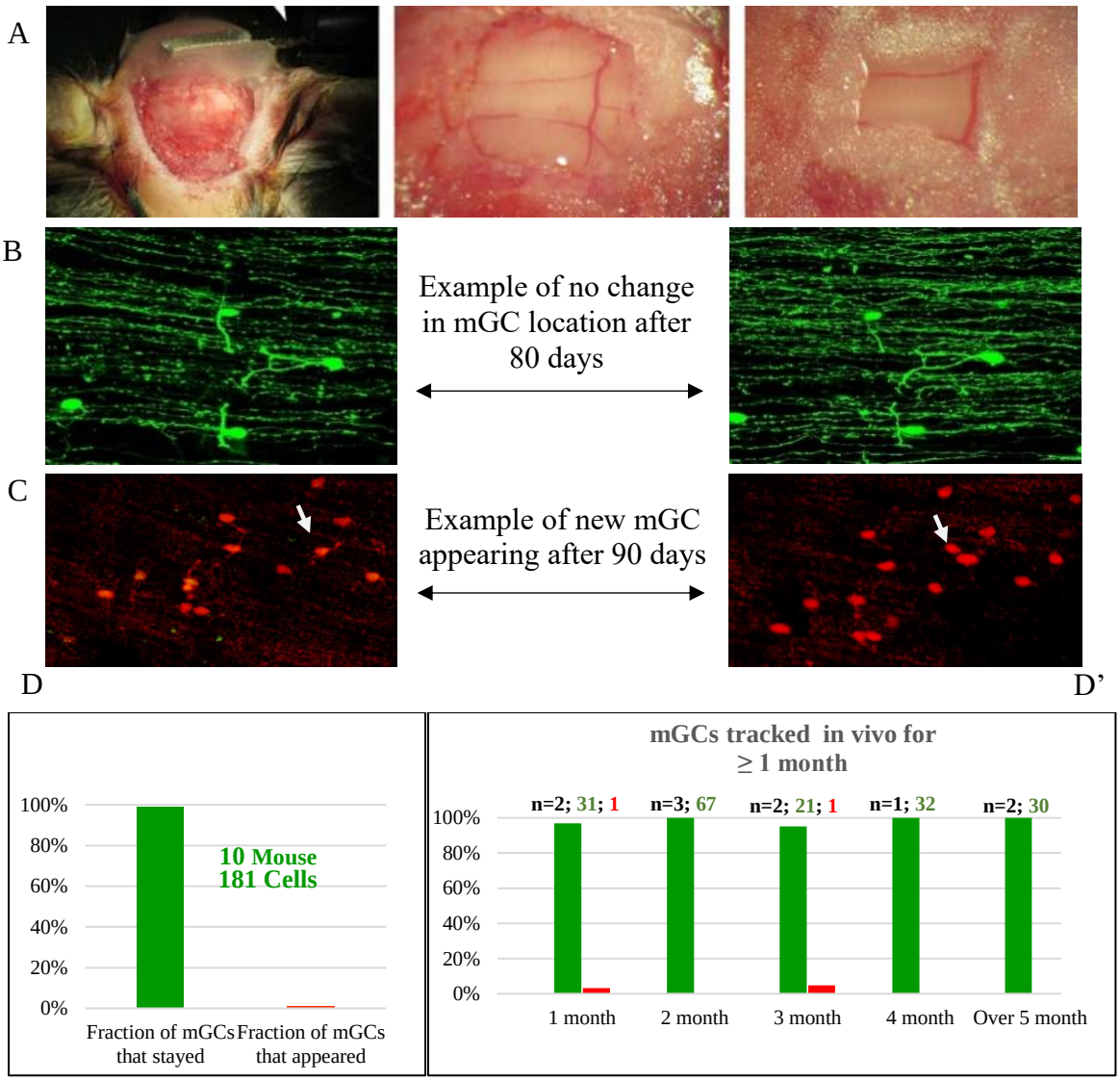

Figure 10.

mGCs are stable in the molecular layer.

A. Surgery to replace small section cranium over cerebellum with glass window to facilitate 2-photon imaging (Nishiyama et al. 2014). **B.** Example z-stack images of mGCs imaged 80 days apart showing that they are in the same 3dimensional position. **C.** One of the only 2 mGCs in 2 different mice (out of 344 cells imaged from 17 mice) which appeared after 90 and 30 days, respectively. The white arrows point to before and after the cells appear for each mice. **D.** For quantification of fraction of mGCs that stayed in their original location, only mGCs tracked for over a month or longer were considered. **D'** the same data from **D** separated by the starting time of imaging to see whether age is a factor in the appearance of the 2 mGCs.

TCGO mice, characterized in chapter 2, were used to track the movement of mGCs *in vivo* over time. A glass window over the cerebellum in place of skull allowed 2 photon imaging of the entire depth of the molecular layer (Fig. 10A). The three dimensional location of mGCs tracked were confirmed by the surrounding parallel fibers in the molecular layer which are also labelled by *mCitrine* (along with mGCs) (Fig. 10B). A total of 344 cells were examined from 17 mice and only 2 cells appeared, none disappeared (Fig 10C). Each of the cells were closely examined and none moved from their original location. Out of the 2 cells that appeared, the appearance was sudden. Therefore, they were either anomalies where two precursors differentiated and migrated late or the mGCs expressed *mCitrine* very late. Late *mCitrine* expression seems more likely because I did not see the 2 cells before the day they appeared, which makes late migration is a more remote possibility.

To further examine whether there is any possibility of mGC appearance with age of the mice (although it is only 2 mGCs), I considered data from mGCs which were tracked for at least one month (Fig 10D) and separated them by the imaging start age (Fig. 10D'). A total of 181 mGCs from 10 mice were separated to 1 month, 2 months ,3 month, 4 month, and 5 or more month of first imaging age of mice. One appeared in mice after 45 days of imaging (started imaging at 1 month) and the other appeared after 90 days of imaging (started imaging at 3 months). There was no correlation between appearance of cell with either age of mice or timeline of imaging (Fig. 10D'). Over 98% of mGCs tracked for over 1 month did not change their location in the molecular layer (Fig. 10D and D'). This

indicates that mGCs are not migrating to the granule cell layer and are integrated in the molecular layer circuitry.

Discussion

Using BrdU proliferation assay and double immunostaining with anti-BrdU and anti-$GABA_AR\alpha6$ antibodies, I have shown that mGCs are born mostly around P8. At P4, when rGCs are at peak birth time, proliferation/differentiation of precursors for mGCs is relatively low. At P8, when the rGC birth time is moderate, mGC birth rate seems to be the highest. At P12, the birth rates of both rGCs and mGCs decline. Overall, my data suggest that compared to rGCs, mGC peak birth time is shifted by only about 4 days. The overall birth timeframe of mGCs seems to lie within the same timeframe as rGCs and mGCs are not the last of the granule cell precursor population to differentiate. Given that differentiation of granule cell precursors and migration to their final destination (at least in the case of rGCs) occur within 31 hours, mGCs are not the last ones to migrate either (Fujita 1967). In addition, it is now known that Bergmann glia closely coordinate the radial migration of granule cells and their position (Araujo et al. 2019; Rahimi-Balaei et al. 2018; Xu et al. 2013). Although yet untested specifically for mGCs, it is possible that mGCs are guided to be in the molecular layer by the Bergmann glia as well. Therefore, mGCs may not be an artefact of defective or late migration, but rather, are intended to be in the molecular layer.

The *in vivo* time-lapse imaging data of tracking mGCs over several months show that mGCs stay in their location in the molecular layer and they are not migrating late towards the granule cell layer. My data set includes mice in which I started tracking mGCs since the animals were 1 month old and out of the 31 mGCs tracked in the younger group, none moved or disappeared and only one appeared. As explained above, the appearance of the 2 new mGCs (in total) is not correlated with age of the mice and maybe an artefact of *mCitrine* expression. If mGCs were migrating late and slowly towards the granule cell layer in mice, as in the case of peripuberal rabbits discussed in the introduction, I would expect the appearance of the two mGCs to be in the younger group and closer to the first day of imaging. Thus, mGCs are stable in the molecular layer and may be integrated into the circuitry. If so, mGCs are potentially functional neurons in the molecular layer; they may be able to fire action potentials and even form synapses with other cerebellar neurons. In such a scenario, they would form a previously unknown circuit because the potential synaptic inputs in the molecular layer are different from those present in the granule cell layer. I have explored this possibility in chapter 4.

Granule cell precursors which differentiate within the same time frame stack their axons in the molecular layer in a similar horizontal plane; the stacking proceeds from closer to the granule cell layer towards the pia with time, or bottom up (Espinosa and Luo 2008). Given my findings that mGCs are born primarily around P8, which is more towards the middle (and later part) of granule cell birth timeline, the parallel fibers of mGCs are presumably stacked mostly at the middle (and upper middle) segments of the molecular

layer (Fig 11). Therefore, mGCs birth time may influence the architecture of their parallel fiber to Purkinje cell dendritic connections.

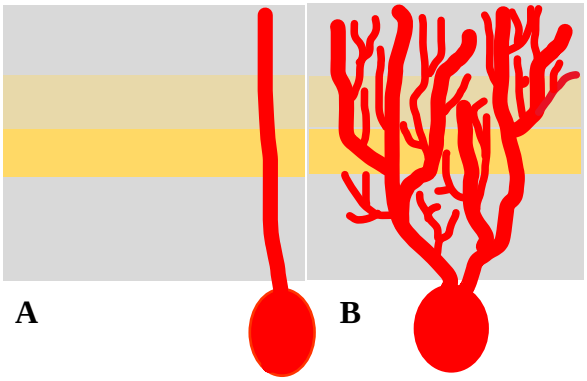

Figure 11.

mGCs stack their parallel fibers in the middle and upper middle part of the molecular layer.

A. Grey region is the molecular layer, Purkinje cell is in red, and parallel fiber stacking is in yellow with color intensity representing fiber density of mGCs. **B.** Same concept as **A**. but rotated 90° in the x-axis and visible PC dendritic arborization.

Chapter 4
Physiological characterization of molecular layer GCs

Abstract

Molecular layer granule cells (mGCs) have been dismissed as ectopic cells which are not part of the cerebellar circuit. Their physiological properties and potential synaptic input have not been explored. I have used whole cell current clamp recordings to measure the input resistance and resting membrane potential of mGCs and found that they are similar to rGCs. One of the characteristics of rGCs in the cerebellum is that they can increase their action potential firing frequency linearly with increasing amplitude of current injections. I have injected increasing current steps to mGCs and recorded their firing frequency. Similar to rGCs, mGCs can increase their firing frequency linearly with current. This suggests that mGCs have the ability to process potentially incoming input at variable intensities. To check whether they receive synaptic input, I recorded from mGCs while electrically stimulating the surrounding molecular layer. The mGCs showed excitatory postsynaptic currents which disappeared after postsynaptic AMPA receptor antagonist was added to the bath solution. Because mGCs are located in the molecular layer, their circuitry is potentially distinct from those of rGCs. The synaptic input to mGCs mostly displayed paired-pulse depression which is characteristic of climbing fiber input (to Purkinje cells).

Introduction

The rGCs in the cerebellum are the smallest and most numerous neurons in the brain and their primary role in the cerebellum is pattern separation of somatosensory inputs (Apps et al. 2018). They have a small soma and an average of four short dendrites of about (Delvendahl, Straub, and Hallermann 2015). The input resistances (R_{in}) of the dendrite and soma of rGCs is very similar (0.5 GΩ to 1.2 GΩ) and their short dendrites makes rGCs electronically very compact (D'Angelo et al. 1995b; Delvendahl et al. 2015). This electrical compactness allows rGCs to process incoming somatosensory input at variable frequencies with high fidelity without signal attenuation dependent on synaptic location and relay it on to Purkinje cells (Apps et al. 2018; Delvendahl et al. 2015).

In vitro, rGCs are able to increase their action potential firing frequency linearly with incremental current injection steps with almost no adaptation (D'Angelo et al. 1995b). The resulting firing frequency versus injected current plot can be fitted to a straight line (D'Angelo et al. 1995b). The membrane properties of mGCs have not been tested.

In the case of rGCs, the synaptic connectivity is well defined and their excitatory input is from mossy fiber terminals (D'Angelo 2018a). However, mGCs have not been considered a contributor to cerebellar circuit and therefore the presence or absence of synaptic input to mGCs have not been tested. The location of mGCs in the molecular layer makes them candidate for a different set of synaptic inputs compared to rGCs.

In this chapter, I used whole cell current clamp technique to measure the R_{in} and resting membrane potential of mGCs. The passive membrane properties of granule cells,

such as resting membrane potential and R_{in}, change significantly as the rGCs mature; the resting membrane potential goes from (approximately, for both set of values) -36 mV to -65 mV and the R_{in} goes from 10 GΩ to 2 GΩ as the rGCs mature from P7 precursors to P15 rGCs (Brandalise et al. 2016). The R_{in} of mGCs is slightly higher than that of rGCs but is not statistically significant; rGCs and mGCs have similar resting membrane potential. I have also found that mGCs are able to fire action potentials and can increase firing frequency linearly with incremental current injections. I used whole cell voltage clamp recordings while electrically stimulating the surrounding molecular layer to test whether mGCs receive synaptic input. They receive excitatory synaptic input, and a majority of the responses show paired-pulse depression reminiscent of climbing fiber input to Purkinje cells (Hashimoto and Kano 1998).

Materials and Methods

Animals

TCGO mice, which are transgenic mouse in which both rGCs and mGCs are labelled sporadically with the fluorescent protein *mCitrine*, of at least 4 month of age were used for all experiments in this chapter. The TCGO transgenic mouse line was introduced in chapter 2. All mice procedures were performed in accordance The Institutional Animal Care and Use Committee (IACUC) and were approved by the University of Texas at Austin Institutional Animal Resource Center.

Sample Preparation

TCGO mice were anesthetized with isoflurane and then killed by rapid decapitation. The cerebellum was quickly removed and submerged in ice-cold cutting solution containing (in mM): 2.5 KCl, 0.5 $CaCl_2$, 7.0 $MgCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 205 Sucrose, and 15 glucose bubbled with 95% O2 and 5% CO2. The right hemisphere was cut off and the cerebellum was mounted on its cut side in a chamber filled with chilled cutting solution. Parasagittal or coronal 200-µm slices were cut using a 7000smz-2 Vibrotome (Campden Instruments), transferred to water bath at ~32°C for 30 min and subsequently stored at room temperature. Artificial cerebrospinal fluid (ACSF) was used for storage, and experiments. Cerebellar sections which had channel rhodopsin injections were prepared, handled, and subsequently patched in minimum light and dark chambers.

ACSF contained (in mM): 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 10 Glucose (~300 mOsm, pH 7.3 when bubbled with Carbogen (5% O_2/95% CO_2). Experiments were performed at 32°C and slices were continuously superfused with ACSF at 2 mL/minute.

Electrophysiological data collection

Cells were selected at random and not restricted to vermis, hemisphere, or lobule. For recording from mGCs, where they had to be identified by *mCitrine* expression, fluorescent light exposure was minimized, and one slice was used for one mGC recording to minimize potential damage from fluorescent light exposure. To record from healthiest cells possible, mGCs from at least 10 µm under the surface were used. For comparison and accounting for any variation due to *mCitrine* expression, data from mGCs (*mCitrine*+), rGCs (*mCitrine*+), and rGCs (*mCitrine*-) were collected (Fig. 12A). For analysis of passive membrane properties, cells were held at 0 pA current and 15 hyperpolarizing steps at 1 pA intervals were applied and the voltage deflection was averaged across 3 traces, for each current step. For analysis of active membrane properties, cells were held at 0 pA and 7 depolarizing steps at 5 pA intervals were applied. For checking whether mGCs receive excitatory synaptic input, the molecular layer near the recorded mGCs was stimulated with a monopolar stimulating electrode to evoke excitatory postsynaptic currents (EPSCs). The ACSF contained 2 µM gabazine, a $GABA_A$ receptor antagonist, to block inhibitory

postsynaptic currents. AMPA receptor antagonist NBQX was applied at 20 µM after 4 to 5 minutes of collecting EPSCs when applicable (Fig 14 B).

Recordings from rGC and mGC soma were made using Multiclamp 700B amplifier (Molecular Devices) and AxoGraph data acquisition software. Patch pipettes were pulled from thin walled borosilicate glass (Sutter Instrument, BF165-120-10) using P-97 Flaming/Brown Micropipette Puller (Sutter Instrument). Patch pipettes had open-tip resistances of 7–12 MΩ; they were fire polished for high GΩ seal and tip wrapped with parafilm to reduce pipette capacitance.

The intracellular solution used for current clamp recordings contained (in mM): 150 K-gluconate, 10 NaCl, 10 K-HEPES (305–310 mOsm, pH adjusted to 7.3 with KOH). The intracellular solution used for voltage clamp recordings contained (in mM): 125 Cs-Methanesulfonate, 10 CsCl, 10 HEPES, and 10 EGTA (305–310 mOsm, pH adjusted to 7.3 with CsOH).

Channel rhodopsin labelling

To label climbing fibers in the right hemisphere, the left inferior olivary nucleus was injected with by adeno-associated virus expressing channelrhodopisin2, AAV-Syn-ChR2-TdTomato (S227), obtained from Dr. Boris Zemelman's laboratory. The labelled climbing fibers are visible via green light (TdTomato) and channelrhodopsin2 can be excited by blue light. Once injected with channelrhodopsin2, the animals were allowed

about 4 weeks before they were used for experiments. For these experiments as well, the ACSF contained 2 μM gabazine. In case of an unlikely possibility that gabazine did not fully block $GABA_A$ receptors, we recorded GABA-mediated inhibitory currents by local application of GABA (200 μM) in the absence of gabazine (n = 6 cells). We confirmed that inhibitory currents were outward in our recording configurations; thus, evoked inward currents are excitatory.

The same virus was used to label mossy fibers by injecting it in the pons on the left side. Green light was used to check for labelled mossy fibers in the molecular layer.

Data analysis

AxoGraph data analysis package was used for data analysis. Analysis of variance (ANOVA), Bartlett's test, and Kruskall-Wallis test were used for statistical analysis of data. Input resistance (R_{in}) was calculated from the voltage changes, from resting holding potential (-65 mV) without any current injection, when depolarizing current steps were injected into the soma (Fig 12 A&B). A graph of change in membrane voltage (Δ mV) of the average of 15 traces was plotted against current injected (pA) and the slope of the graph from 0 pA to 10 pA was used to calculate the R_{in} of each group of granule cell types (Fig 12 D, arrow). Granule cells do not always show a linear voltage versus current relationship with increased hyperpolarizing steps and some authors use the slope at 0 pA from the curve. For my data, I selected the range (0 to 10 pA) in which I observed the relation to be linear for most cells in all three groups. Kruskall-Wallis test was used instead of ANOVA.

Bartlett's test is to test for the homogeneity of variance among groups in a data set and is applicable when the data points within each group show a normal distribution. At first glance, the data points in each three groups for both R_{in} and resting membrane potential seem to have a normal distribution (Fig 12 D, E). But the p-value of Bartlett's test for both R_{in} and resting membrane potential showed that the variance was different among the three groups.

Action potential firing frequency was calculated by plotting the firing rate over the first 200 ms when injected with current at 7 steps of 5 pA increments starting at 0 pA holding current. The firing frequency change with current intensity was also reported as linear slope factor (LSF) and the units are Hz. pA^{-1}. The LSF was calculated for each cell from the slope of the current-frequency plot starting at one step before the threshold current to maximum current versus frequency data point (Fig 13 D). The variability among groups for LSF was not similar ($p = 0.002$, Bartlett's test) and so the Kruskall-Wallis test was used. The LSF is useful because the threshold current and maximum frequency varies substantially across granule cells and this normalizes the measure for granule cell excitability (Brandalise et al. 2016). The input threshold was reported as the current step right before the one at which the cell fired action potentials. The action potential threshold was the membrane voltage at which point the voltage upshot rapidly to fire an action potential. Kruskall-Wallis test was used for statistical analysis of LSF, input threshold, and action potential threshold.

Data from electrical stimulation in molecular layer to check for synaptically activated responses from mGCs was manually counted using AxoGraph data analysis software. Data from experiments utilizing channelrhodopsin2 were also analyzed manually using AxoGraph.

Results

The physiological properties of mGCs are unknown and their location in the molecular layer makes it very difficult to identify them in live tissue. The TCGO mouse expresses *mCitrine* sporadically in granule cells and so allows the identification of mGCs in the molecular layer via *mCitrine* expression which fluoresces yellow/green under blue light (Huang et al. 2013). However, the sporadic expression of *mCitrine* means that rGCs have *mCitrine* positive and negative populations. For the physiological characterization of mGCs, I have used both *mCitrine* positive and negative rGCs, as control and comparison to account for any potential variability. Therefore, the cells I recorded from and reported are mGCs (*mCitrine* +), rGCs (*mCitrine* +), and rGCs (*mCitrine* -) (Fig 12 A).

The voltage-current relationship was obtained in each cell (Fig. 12 B), and the input resistance (R_{in}) was measured as the slope of the linear regression line (Fig. 12 C). The R_{in} for both rGCs (*mCitrine* -) and rGCs (*mCitrine* +) showed cell-to-cell variation with R_{in} =1.65 ± 0.85 GΩ (n =11 cells) and R_{in} = 2.30 ± 0.98 GΩ (n =7 cells) respectively, as indicated by the relatively large standard deviation compared to the mean (Fig 12 D, E).

But the variation in R_{in} was small in mGCs (*mCitrine* +) with R_{in} =2.42 ± 0.39 GΩ (n =12 cells) (Fig 12 D).

For $R_{in,}$ the p-value for the Kruskal-Wallis test is 0.059. Therefore, there is no significant difference in the R_{in} among the three groups. The resting membrane potential was not significantly different among the cell-types either (Fig. 12 F) The p-value for the Kruskal-Wallis test is 0.537. The values for both R_{in} and resting membrane potential indicate that mGCs are as mature as rGCs (Fig 12 E, F).

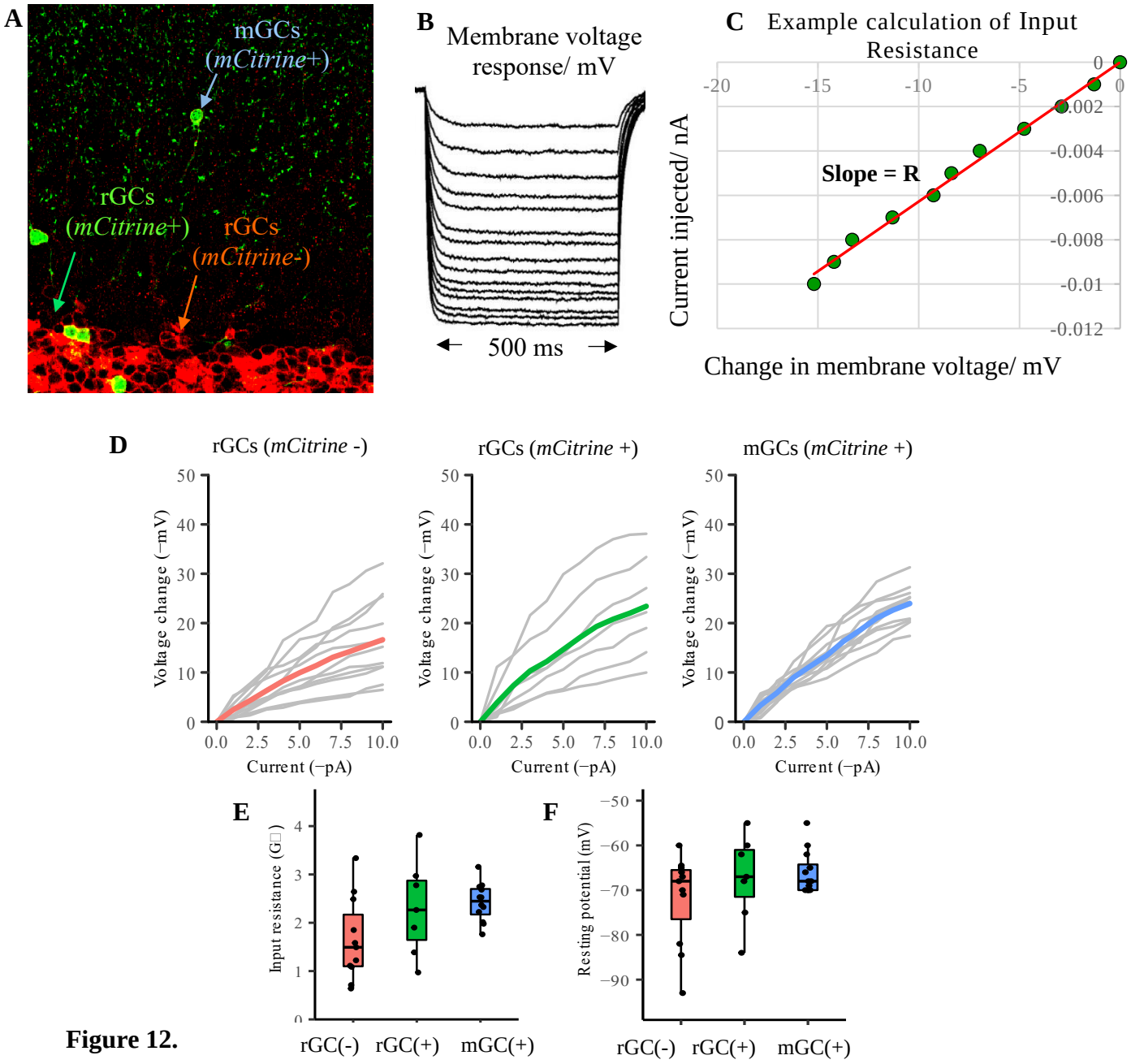

Figure 12.

mGCs have similar input resistance and resting membrane potential to rGCs.

A. The three cell types which were recorded from. **B.** Example of a granule cell's membrane voltage deflection as response to current injections. **C.** Example of input resistance calculation from slope of Δ mV vs. injected current graph, 0-10 pA was used. **D.** Δ mV vs. current curve of each cell (in grey) and average (in bold color) for all three cell groups. **E. & F.** Average input resistance and resting membrane potentials, respectively for each cell type. Black dots represent individual cells.

I tested whether mGCs can fire action potentials and if the firing frequency linearly increases with stimulus intensity, as is the case for rGCs. Both *mCitrine* positive and negative rGCs fire action potential with increasing frequency as more current injected, which is consistent with previous studies. Molecular layer granule cells also fire action potentials and the frequency increases with current (Fig 13 A, B, C).

Granule cells, both rGCs and mGCs, have slight variability in their R_{in} (Fig. 12 E) which leads to slightly variable input threshold within group, which is the minimum current needed to elicit an action potential (Fig 13 F, black dots represent individual cell data). The input threshold among the three groups was not different ($p = 0.438$, Kruskal-Wallis test). The action potential threshold is not statistically significant either ($p = 0.332$, Kruskal-Wallis test). The LSF (Hz. pA^{-1}) was calculated from the slope of the frequency-current graph of each cell (Fig 13 D). LSF is useful because the threshold current and maximum frequency varies substantially across granule cells and LSF normalizes the measure for granule cell excitability. There was no statistical difference in the LSF of mGCs, rGCs (*mCitrine*+), and rGCs (*mCitrine*-) ($p = 0.289$, Kruskal-Wallis test) (Fig 13 G). Therefore, mGCs discharge action potentials in a very similar way to rGCs and they may be part of a synaptic circuit.

I tested whether they receive excitatory input. I held mGCs via whole cell voltage clamp mode and electrically stimulated the surrounding molecular layer with paired-pulses at 50 ms apart (Fig 14 A). A fraction of the mGCs showed a response (25 out of 60 mGCs randomly selected across sections) to the paired-pulse stimulation (Fig 14 C).

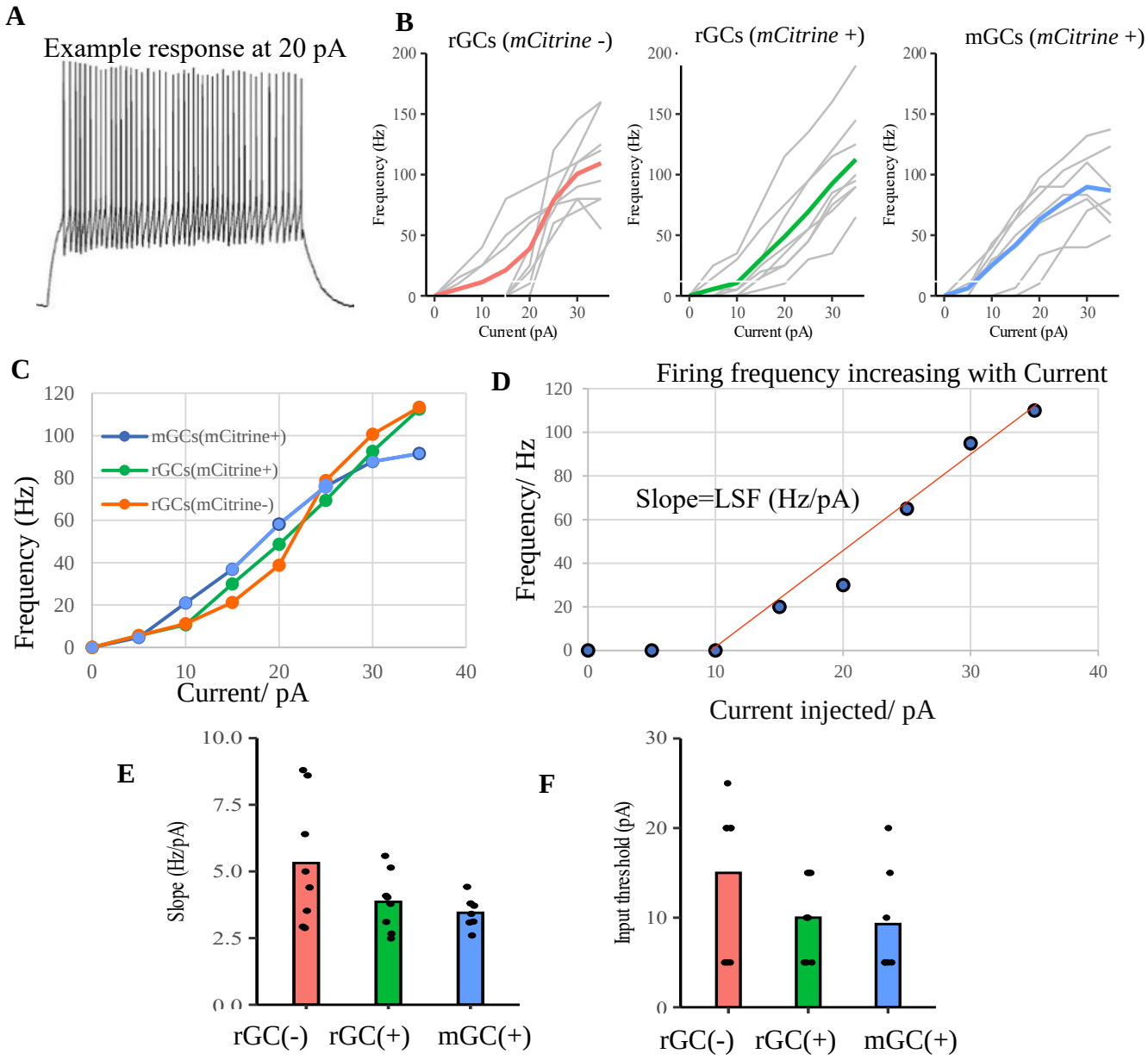

Figure 13.

mGCs can increase firing frequency similar to rGCs.

A. Example of mGC firing at high frequency when injected with 20 pA. **B.** Firing frequency vs. current injected curve of each cell (in grey) and average (in bold color) for all three cell groups. **C.** Average firing frequency vs. current curve of the three cell types plotted together. **D.** Example of LSF calculation from slope of firing frequency vs. current injected graph. **E.** Average LSF of the three cell types. **F.** Average input threshold for all three cell types. Black dots in E & F represent individual cell.

Addition of an AMPA receptor antagonist, NBQX, blocked the inward current responses suggesting that the responses were excitatory and synaptic (Fig 14). Among the 25 mGCs that responded, the majority (23 mGCs) showed paired-pulse depression (Fig 14 D). The nature of climbing fiber input to Purkinje cells is paired-pulse depression and the majority of evoked responses on mGCs being paired-pulse depression suggest that the source of input is mostly climbing fibers.

The molecular layer has three sources of excitatory input: climbing fibers, parallel fibers, and occasional mossy fibers. To identify the source of input to mGCs I used optogenetics and started with labelling the climbing fibers with channelrhodopsin2 (Fig 15A). Whole cell patch clamp recordings of mGCs in conjunction with stimulation of surrounding channelrhodopsin2 labelled climbing fibers resulted in only one out of 72 mGCs showing a response. Furthermore, only the first blue-light pulse evoked the response but the second light pulse (illuminated 70 ms after the first light pulse) did not.

Mossy fibers terminate as rosettes in the granule cell layer but occasionally they extend to the molecular layer and it has been hypothesized that these make synaptic connections with mGCs (Berciano and Lafarga 1988). Mossy fibers can show both paired-pulse depression and facilitation (Chabrol et al. 2015). I used a similar strategy using channelrhodopsin2 to test whether mGCs receive synaptic input from mossy fibers. I could not find any mossy fiber in the molecular layer although mossy fibers were densely labelled in the granule cell layer. Therefore, my optogenetic stimulation did not provide conclusive result regarding the input source of mGCs.

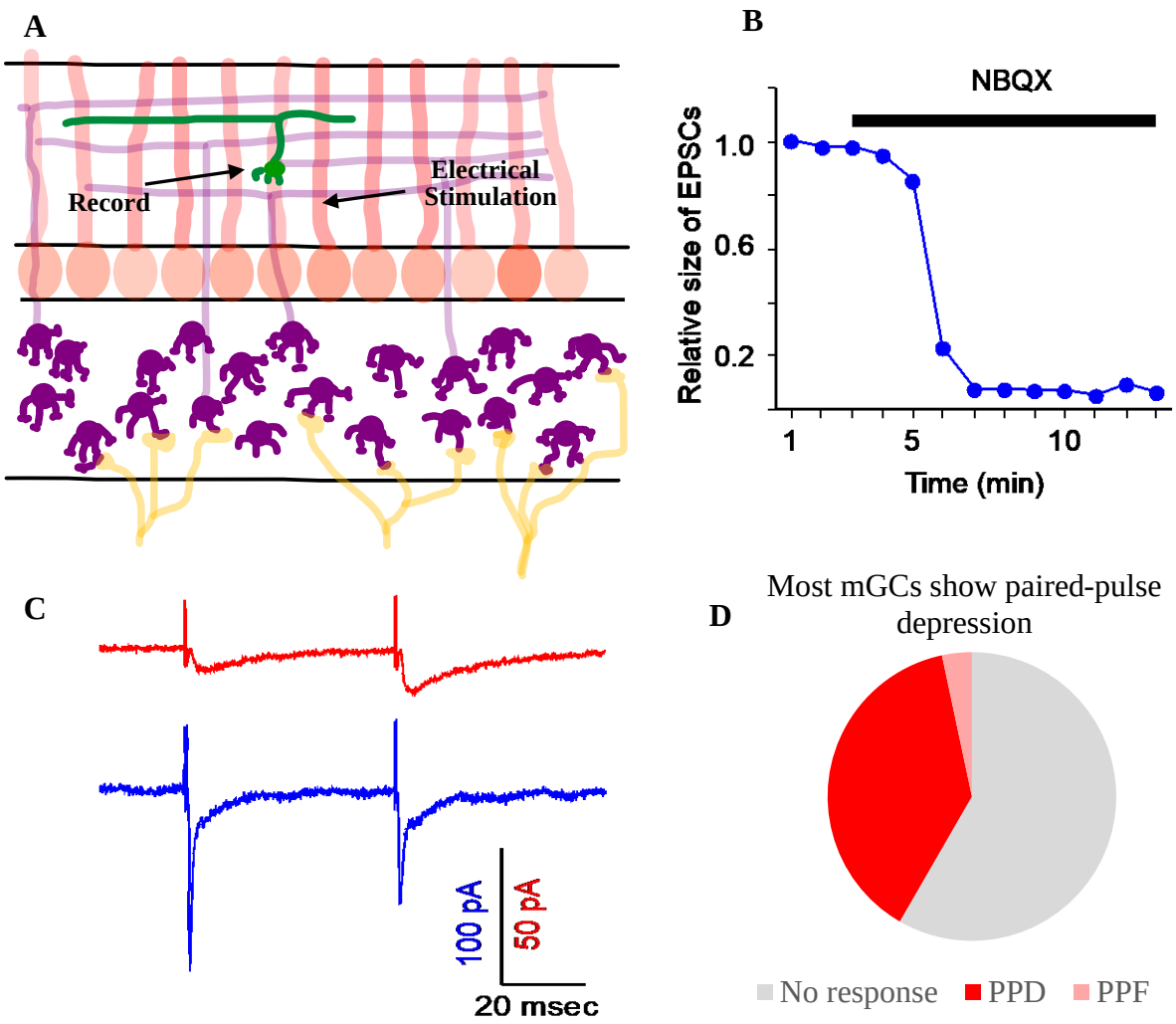

Figure 14.

mGCs receive excitatory synaptic input.

A. Schematic diagram showing how mGCs were patch in whole-cell voltage clamp configuration and the surrounding molecular layer was stimulated with a bipolar stimulating electrode. The stim. electrode was moved around within the molecular layer until a response was seen in the mGC. **B.** Example of mGCs showing the evoked responses are synaptic. The AMPA receptor antagonist, NBQX, was added after 4 minutes of electrical stimulation once the mGC showed a response. Addition of NBQX results in gradual disappearance of excitatory currents. **C.** Examples pf paired-pulse facilitation (red) and paired-pulse depression (blue) recorded from mGCs. **D.** Pie-chart showing the fraction of mGCs which did not show response (grey), fraction of mGCs which showed paired-pulse depression (red), and fraction of mGCs which showed paired-pulse facilitation (salmon).

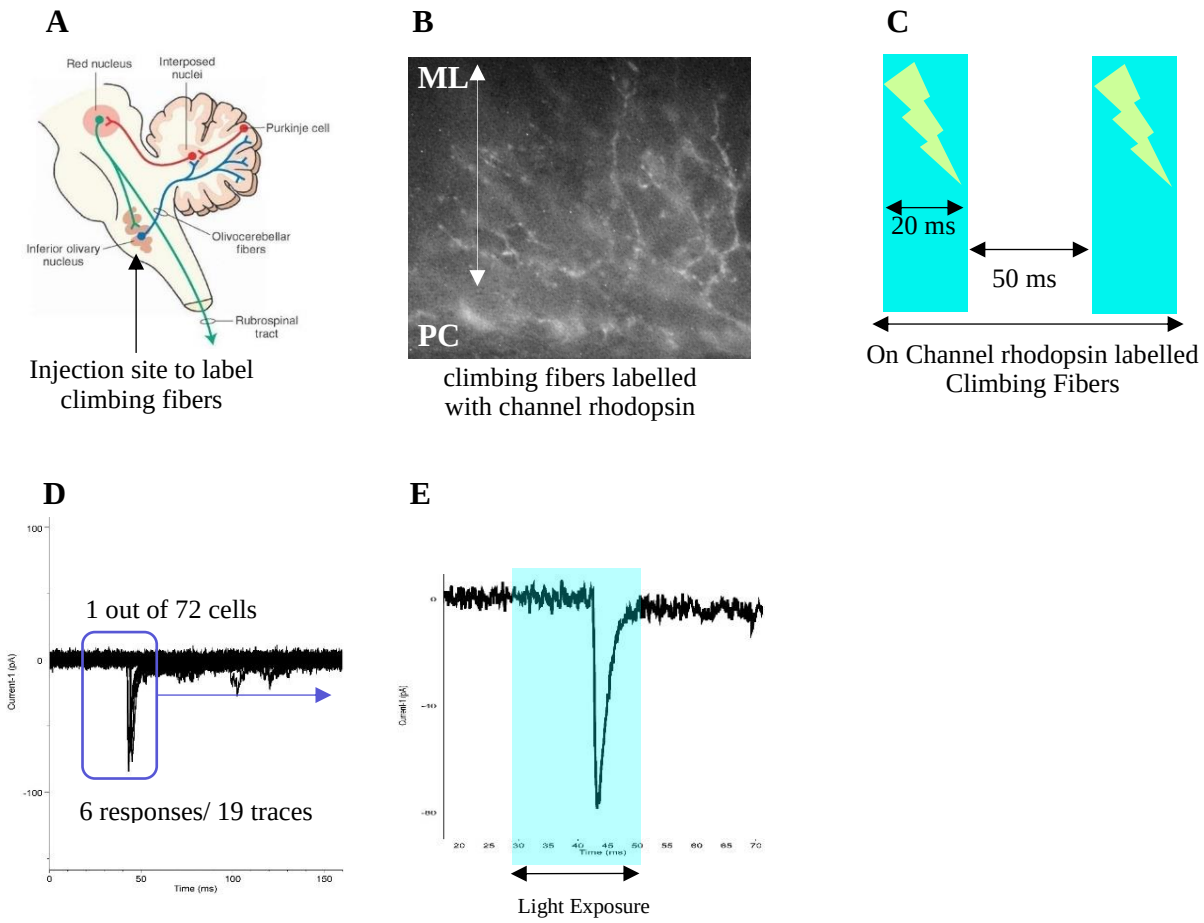

Figure 15.

Rare but possible climbing fiber input to mGCs.

A. adeno-associated virus expressing channelrhodopisin2, (S227) AAV-Syn-ChR2-TdTomato, was injected into the left inferior olivary nucleus. **B**. Example cerebellar slice from the right hemisphere showing climbing fibers under fluorescent green light (TdTomato, red signal). **C.** protocol used to shine blue light to excite climbing fibers with channelrhodopsin2. D. Only 1 out of 72 cells showed a repeated response to the first light flash only. E. One of the traces from D with the light exposure time superimposed on it.

Discussion

In the past mGCs were studied using nonspecific staining methods, such as Golgi staining and the few identifiable mGCs in subpial clusters showed fewer number of dendrites (on average) and smaller somas (Berciano and Lafarga 1988). This led to the hypothesis that mGCs are stuck in their migratory pathway in an immature state (Berciano and Lafarga 1988). It is now known that immature granule cells have higher resting membrane potential (~36 mV), much higher membrane resistance (~ 10 GΩ), and are not able to fire action potentials (lack sodium spikes) (Brandalise et al. 2016). I have shown that mGCs do not show the physiological characteristics of immature granule cells; their resting membrane potential (~65 mV), membrane resistance (~ 2.4 GΩ), and ability to fire action potentials at high frequencies (over 100 Hz) are similar to those of mature rGCs. This result is consistent with findings from chapter 3 where I show that mGCs express molecular marker for mature rGCs ($GABA_AR\alpha6$).

The variance for the mGCs data on some of the physiological properties (R_{in} and LSF) is much less than the two other groups, rGCs (*mCitrine* +) and rGCs (*mCitrine*-). A possible reason maybe because only mGCs which express *mCitrine* were patched and the expression may be for a specific subgroup of mGCs instead of being random.

Majority of mGCs which receive excitatory synaptic input, show paired-pulse depression which is characteristic of climbing fiber input. Further examination of the source of input by targeting climbing fibers via channelrhodopsin2 did not yield any conclusive result. Inferior olive injection of the virus does not always label all the climbing fibers in a particular region and *mCitrine* labels mGCs sparsely; these two factors make

finding mGC adjacent to a climbing fiber difficult and they may not be close enough to be synaptically connected even if it appears to be so by visual inspection under a light microscope. Depending on the slice and angle of cutting, mGCs or climbing fibers (or both) may be partially cut off which renders them unhealthy and therefore potentially unresponsive.

Channelrhodopsin2 injection to label mossy fibers in the molecular layer did not yield any result. This outcome is not surprising because the pons on one side was injected and only a fraction of mossy fibers enters the cerebellar cortex via the pons. The mossy fibers in the white mater and granule cell layer were intensely labelled but I could not find any mossy fiber going up into the molecular layer. Therefore, mossy fiber innervation to the molecular layer is very rare and so it is unlikely that mossy fibers can provide input to all mGCs which are one of the most abundant cells in the

The exact source or sources of synaptic input to mGCs is still ambiguous. The excitatory axons present in the molecular layer are depicted in figure 16 below. Given that most of the responses are paired-pulse depression and only a small fraction (2 out of 25 mGCs which showed a response) show paired-pulse facilitation, it is possible that mGC receive input from climbing fibers, parallel fibers, and the occasional mossy fiber in the molecular layer.

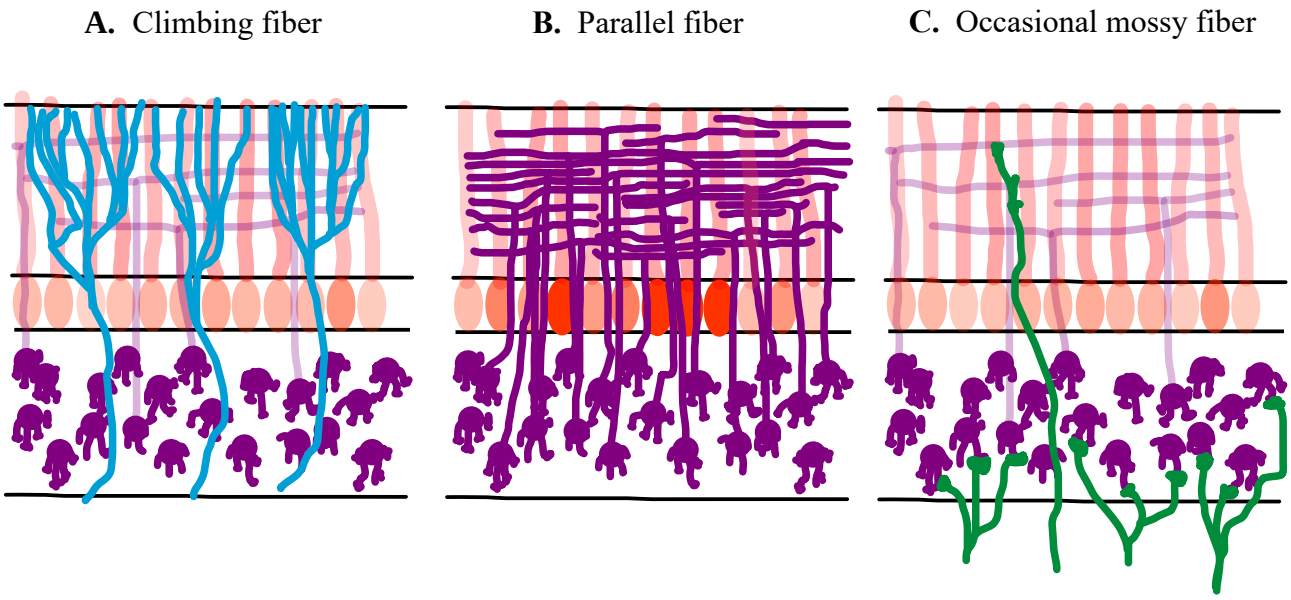

Figure 16.

Potential sources of excitatory synaptic input to mGCs.

A. Climbing fibers are strong glutamatergic axons and they innervate the molecular layer extensively. They show paired-pulse depression and some spillover transmission due to the large amounts of glutamate released. **B.** Parallel fibers also innervate the molecular layer extensively and they seem to synapse onto most of the cell types in the molecular layer. They generally show paired-pulse facilitation. C. Occasionally, mossy fibers innervate the molecular layer. They show both paired-pulse depression and paired-pulse facilitation.

Chapter 5

Discussion

Summary

In this dissertation, I use immunohistochemical analysis, two-photon imaging, and electrophysiology to reevaluate molecular layer granule cells, mGCs, and their potential role in the cerebellar circuitry. I show that mGCs are possibly the most numerous cells in the molecular layer because they make up one third of the molecular layer cell population. They express the molecular marker specific for mature granule cells, $GABA_AR\alpha6$, and this is supported by their resting membrane potential which is akin to mature rGCs. The mGCs are born within the same time frame as rGCs, a few days delayed, but not at the end and mGCs are stable in the molecular layer circuitry. The input resistance and firing properties are similar to those of rGCs. In addition, mGCs receive excitatory postsynaptic input. Given that mGCs are in the molecular layer, they may be receiving synaptic inputs different than those to rGCs. The synaptic input to mGCs was mostly paired-pulse depression which is characteristic of climbing fiber input. My data suggest that mGCs are a part of the cerebellar circuitry. The exact synaptic input source(s) and functional significance is yet to be determined.

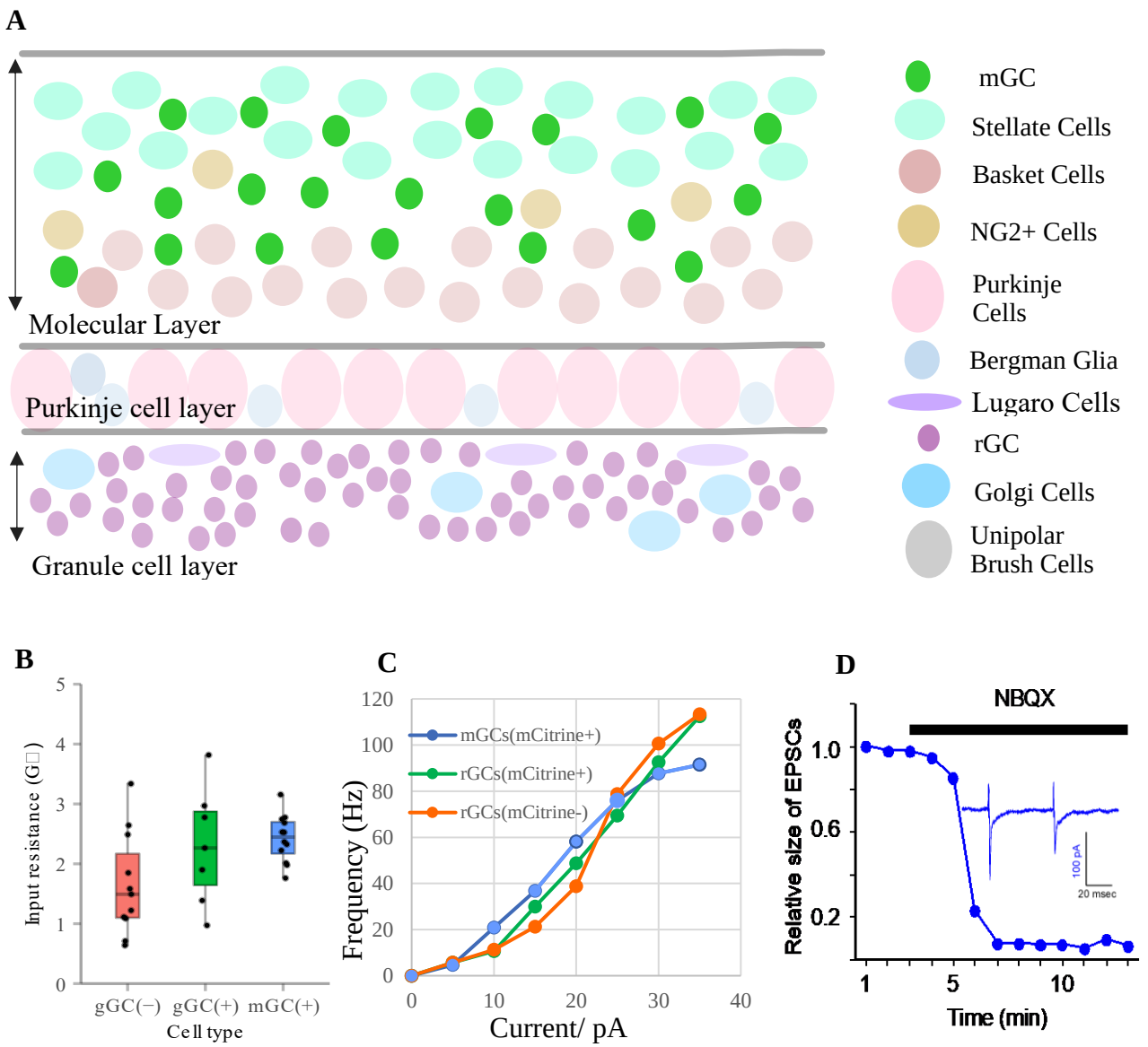

Figure 17.

Summary of old versus new information and major findings.

A. Schematic representation of the cell types and relative density of the cerebellar cortex. The faded out cells represent the cells which are known to be a significant part of the cerebellar cortex, bright green cells represent mGCs which were thought to be ectopic, but I show that they make up a third of the molecular layer. **B.** The input resistance of mGCs is in the GΩs and not statistically different from rGCs. **C.** mGCs can increase their firing frequency linearly with increasing current, similar to rGCs. **D.** mGCs receive synaptic input and most of them show paired-pulse depression, which is indicative of climbing fiber input.

Ectopic neurons are found in the normal adult brain and minor defects in migration during development is generally attributed for their occurrence; the dentate gyrus, entorhinal cortex, and the cerebellar molecular layer are some of the known locations of ectopic neurons. They are generally not quantified or well-characterized because they are thought to be very few in numbers and not a significant contributor to the local circuitry. An example is the small number of granule cells in the hilus of the normal mouse dentate gyrus. These ectopic granule cells are very close to the molecular layer, where they are usually found. But they have only been studied in a disease context in which their morphological and physiological properties similar to those in the molecular layer. Their synaptic connections are the same as the granule cells in the molecular layer. Given the small numbers and same circuit placement, granule cells of the hilus are considered ectopic and not a dominant contributor to the hippocampal circuitry. The story is similar for the pyramidal neurons of the entorhinal cortex layer IV. My results suggest that the story may be different for mGCs, which have long been ignored as ectopic neurons in the cerebellum. In this final chapter, I will briefly discuss my findings about mGCs and what they might mean in a larger context.

mGCs may be the most abundant neurons in the molecular layer

Granule cells express combinations of the $GABA_AR\alpha$ subunits 1 through 6 as they mature (Laurie et al. 1992). For this dissertation, I tested for the receptor subunit α6 which is expressed in mature granule cells only. I did not test for the immature molecular marker.

Although it is unlikely, considering the high fraction of mature mGCs present, it is possible that immature mGCs may also be present in the adult cerebellum.

Using the mature granule cell marker to quantify the fraction of mGCs which make up the molecular layer, I show that mGCs are one third of the total cell population and so the remaining 2/3rds of the molecular layer is made up by inhibitory interneurons (a combination of basket and stellate cells) and few NG2 positive glial cells. Therefore, it is possible that mGCs are the most abundant cell type of the molecular layer. My dissertation work is the first comprehensive analysis of mGCs, which might lead to the revision of cerebellar synaptic circuitry.

mGCs are not a result of late birth and consequent migration defects

The process of granule cell production begins in the embryonic stages, as early as E11, and all granule cells are produced from *Math1* expression progenitor population of the external granule cell layer (Ben-Arie et al. 1997; Wang et al. 2005). Their proliferation, differentiation, and migration are guided by an orchestrated array of signaling molecules and physical scaffolds. For example: *Shh*, responsible for granule cell precursor proliferation, also induces the maturation of radial glia to Bergmann glia, which are partially responsible for the granule cell maturation process and granule cells use the Bergmann glial processes as scaffolds during migration (Espinosa and Luo 2008; Dahmane and Ruiz-i-Altaba 1999; Xu et al. 2013).

BrdU assay shows that the peak BrdU positive mGCs was when mice were injected with BrdU at P8 and this is within the normal range for granule cell precursor proliferation. Granule cell precursor proliferation is dependent on *Shh* secretion by Purkinje cells which stop secreting *Shh* around the time proliferation stops (P14 to 18) (Espinosa and Luo 2008). If mGCs were the last granule cells to exit the cell cycle, all mGCs would be BrdU positive at 2 months regardless of injection timing, or P12 injection would have the most BrdU positive mGCs. This is not the case and there is a clear peak at P8, while it is significantly lower at P4 and P12. This indicates that mGCs are not a result of the last wave of granule cell birth which could not complete their migration to the destination.

In addition, Bergmann glial fibers have been known to act as physical scaffold to guide granule cells to their destination for a long time, but more recent studies suggest that they play a more active role in the process via molecular cross-talk with the migrating granule cells (Araujo et al. 2019). Therefore, it is conceivable that mGCs are guided by Bergmann glial fibers to be in the molecular layer.

mGCs may have different synaptic input compared to rGCs

The dendrites of mGCs are in the molecular layer and so any potential synaptic connections, if present, are from axons present in the molecular layer. This makes mGCs in a unique position of receiving synaptic inputs from very different source(s) compared to rGCs. Excitatory axons present in the molecular layer are climbing fibers, parallel fibers, and occasional mossy fibers. The molecular layer is densely packed with parallel fibers

and they make synaptic connections with every cell type present in their path (D'Angelo 2018a). Climbing fibers are very strong glutamatergic axons which make synaptic connections with Purkinje cells but have spillover mediated transmission to both inhibitory interneurons of the molecular layer (D'Angelo 2018a).

Data from electrical stimulation experiments, shown in chapter 4, suggest that at least some mGCs are most likely receiving climbing fiber input because they show the typical paired-pulse depression response shown by climbing fibers. As described in chapter 4, experiments using channelrhodopsin2 labelled climbing fibers were inconclusive.

Results from electrical stimulation experiments also showed a small fraction of mGCs showing paired-pulse facilitation which are typical parallel fiber responses. This outcome is not surprising given that the molecular layer is very densely packed with parallel fibers. However, mossy fibers input to rGCs can be paired-pulse depression or paired-pulse facilitation (Chabrol et al. 2015). Since, some mGC clusters near the pia have been reported to be innervated by mossy fiber terminals (Lafarga and Berciano 1985), I cannot exclude the possibility that mossy fibers are the major input source of mGCs. However, I think that the possibility is remote because the same study quoted above (Lafarga and Berciano 1985) did not find mossy fiber innervation to mGCs dispersed in the molecular layer. Besides, mossy fiber innervation to the molecular layer is rare, which is likely insufficient to provide input to most mGCs. All factors taken together, climbing fibers remain to be a strong candidate for mGC input.

Functional consequences of the potential mGC circuits

One of the potential sources of synaptic input to mGCs are climbing fibers. During Pavlovian eyelid conditioning, climbing fibers bring in the error signal (air puff to the eye) to the Purkinje cells (De Zeeuw 2020). The incoming climbing fibers, or error incoming pathways, are restricted to specific microzones; for example, the climbing fibers responsible for eyeblink responses are restricted to a region in the lobule HVI (Halverson et al. 2018; Mostofi et al. 2010). The climbing fiber input to the cerebellar circuit seems to be restricted to the olivo-cortico-nucleo-olivary (OCNO) circuit. This means that specific error signals responsible for the teaching portion of cerebellar associative learning are restricted within the OCNO circuit.

In a larger context, learning does not occur in discrete segments. At a given time, the cerebellar circuitry is most likely trying to learn several associations simultaneously and so there needs to be some level of communication between the error signals. So far, there are no known methods of communication between the OCNO loops within the cerebellar circuit. If mGCs receive climbing fiber input, it would be a way for the OCNO loops to communicate because the parallel fibers of mGCs, like those of rGCs, extend in either direction in the molecular layer and are also long. The parallel fibers from mGCs most likely synapse onto the Purkinje cells, similar to rGCs, because all granule cells extend their axons towards Purkinje cell dendrites during differentiation. In such a scenario, mGCs may be the means of communication between the OCNO loops and acting as a coordinator between various error signals.

The second possibility of synaptic input to mGCs are parallel fibers from rGCs. In this scenario, mGCs would act as a relay of information between the rGCs and Purkinje cells and add a short time lapse in the information flow, depending on the length of the mGC axons. On average, the action potential propagation speed in parallel fibers is 0.2 to 0.3 m/s, depending on the position of the parallel fiber in the molecular layer (Vranesic et al. 1994). Parallel fibers are difficult to trace but the average approximate length of parallel fibers is about 10 mm, with about 5 mm in either direction after bifurcation Given the range of speed and average length, an mGC would add a time delay of 17 ms to 25 ms of signals from rGCs to Purkinje cells. In this scenario, mGCs would add 17 ms to 25 ms delay in a small subset of somatosensory information to Purkinje cells.

The third possibility is mossy fibers synapsing on to mGCs. In this scenario, the cerebellar circuitry would not be novel because rGCs receive input from mossy fibers.

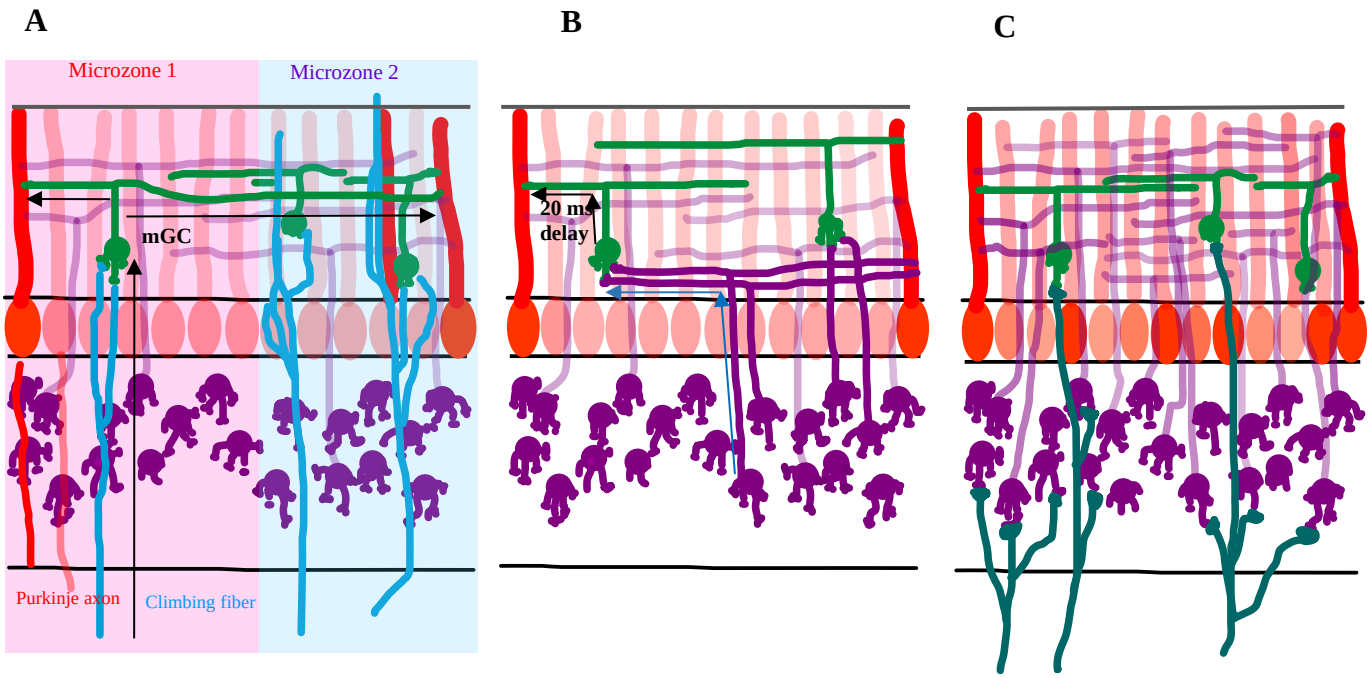

Figure 18.

Schematic representation of the potential mGC input scenarios.

A. If mGCs receive climbing fiber input: the olivo-cortico-nucleo-olivary (OCNO) loops of different microzones can communicate. Black arrows point to how climbing fiber input to mGC in microzone 1 can be transferred to Purkinje cell dendrites in both microzone 1 and 2. Microzones are shown by overlayed colored boxes (pink and blue). **B.** If mGCs receive rGC input: the mGCs would act as relay of information to the Purkinje cell. Blue arrows show how information from rGCs reach mGC and black arrows show how the mGC fibers add an additional 20 ms for the information to reach the Purkinje cell. **C.** If mGCs receive mossy fiber input: The overall circuitry would not be changed.

Future directions

The output of mGCs will allow for a broader understanding of their place in the cerebellar circuitry. Morphological studies on subpial mGC clusters show that they have bifurcated parallel fibers which innervate Purkinje cell dendrites (Berciano and Lafarga 1988). Tracking the parallel fibers of *mCitrine* labelled mGCs in the TCGO mouse is challenging because the molecular layer is packed with *mCitrine* labelled parallel fibers from rGCs. Filling mGCs, a different depths in the molecular layer, with a red fluorescent dye in the TCGO mouse would give us an idea of the output target(s) of mGCs.

Inhibitory input to the mGCs were not explored in this dissertation and all experiments investigating synaptic input had a $GABA_A$ receptor antagonist, gabazine, in the bath solution. It is conceivable that mGCs receive inhibitory inputs from the basket and stellate cells of the molecular layer. The next step will be to check whether mGCs receive inhibitory input. If mGCs receive inhibitory input, this would mean that they have a more complex role than the simplified signal transmitters they have been explained as in the section above.

For any neuron to be relevant, the most important step is to show whether they are actively participating while the animal is engaged in any behavior. Calcium imaging of mGCs *in vivo*, while the mouse is engaged in a particular task, would be a good indicator of whether mGCs are behaviorally involved.

Conclusion

The main finding of this study is that mGCs are possibly the major component of the cerebellar molecular layer. These cells are not the last ones of the granule cell precursors to differentiate and they may be destined to be in the molecular layer. Molecular layer granule cells receive excitatory synaptic input. Given the location of mGCs and the results from electrical stimulation, mGCs may form a novel excitatory circuit involved in error signal coordination in the cerebellar cortex.

Our understanding of the cerebellar circuitry is continuing to evolve with recent findings about new cell types and new synaptic associations of well-studied cells; one such example is the inputs of Purkinje cell collaterals (Guo et al. 2016; Hirono et al. 2012; Ruigrok et al. 2015; Schilling et al. 2008; Witter et al. 2016). Molecular layer granule cells may also become a new addition to the cerebellar circuitry, and this work is the first step towards understanding their potential role.

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